

TITLEPLASMIN INHIBITORS FROM THE AUSTRALIAN BROWN SNAKE PSEUDONAJA TEXTILIS TEXTILISFIELD OF THE INVENTION

THIS INVENTION relates to anti-fibrinolytic agents and in particular, novel plasmin inhibitors having reduced propensity for causation of rebound thrombosis. The present invention also relates to amino acid sequences and nucleotide sequences encoding the novel plasmin inhibitors as well as to methods of producing these inhibitors and pharmaceutical compositions containing same.

BACKGROUND OF THE INVENTION

The blood loss associated with major forms of surgery has in the past been compensated by replacement therapy, which may involve fresh frozen plasma, fresh whole blood and platelet concentrates. With recent awareness of a variety of blood borne viral infections (Hepatitis B and C, and human immunodeficiency virus, HIV), the need to reduce blood loss during surgery is a major priority. Further anxiety has been generated within National Blood Transfusion Services concerning infectivity with agents related to Bovine Spongiform Encephalitis (BSE) and Creutzfeldt-Jacob's Disease (CJD) for which there is no reliable assay at the present time.

It has been established (Royston, 1990, *Blood Coagul. Fibrinol.* 1:53-69; Orchard *et al*, 1993, *Br. J. Haemat.* 85:596-599) that unfettered fibrinolytic activity via the plasminogen-plasmin pathway contributes to haemorrhage and that a plasmin inhibitor such as aprotinin helps alleviate blood loss. This seems to suggest that plasmin-mediated digestion of fibrin clots and components of the coagulation system may be of primary importance as a contribution to this haemorrhagic state (Orchard *et al*, 1993, *supra*).

The use of aprotinin during cardiopulmonary bypass (CPB) surgery is now commonplace (Royston, 1990, *supra*; Orchard *et al*, 1993, *supra*). In particular, Orchard *et al* (1993, *supra*) have demonstrated that the bovine source

inhibitor aprotinin, as the active substance in the medicament Trasylol™, reduces blood loss in CPB patients by neutralisation of plasmin activity and does not affect platelet activity. This latter finding has been confirmed by other investigators (Ray and March, 1997, *Thromb. Haemost.* 78:1021-1026).

5 Aprotinin is a well-investigated serine protease inhibitor, or 'serpin'. It comprises 58 amino acids and acts to inhibit trypsin, α -chymotrypsin, plasmin as well as tissue and plasma kallikrein (Fritz and Wunderer, 1983, *Drug Res.* 33:479-494; Gebhard *et al*, 1986 In "*Proteinase Inhibitors*", Barrett and Salvesen (eds.), Elsevier Science Publications BV pp 374-387). Aprotinin has
10 also been found to react with thrombin and the plasminogen activators (tPA and uPA) (Willmott *et al*, 1995, *Fibrinolysis* 9:1-8).

 Recent studies have shown that semi-synthetically generated homologues of aprotinin that contain other amino acids in place of lysine at position 15 of the amino acid sequence have a profile of action and specificity of
15 action which differ distinctively from those of aprotinin (US Patent No 4,595,674; Wenzel *et al*, 1985, In "*Chemistry of Peptides and Proteins*" Vol. 3). Some of these semi-synthetic aprotinin homologues have, for example, a strongly inhibiting action on elastase from pancreas and leucocytes. Other aprotinin homologues with arginine at position 15, alanine at position 17, and serine at
20 position 42, are characterised by an inhibitory action which is distinctly greater than that of aprotinin on plasma kallikrein (*cf.* WO 89/10374).

 Reference also may be made to US Patent No 5,576,294 (Norris *et al*) which discloses human protease inhibitors of the same type as aprotinin. In particular, there is disclosed variants of human Kunitz-type protease inhibitor that
25 preferentially inhibit neutrophil elastase, cathepsin G and/or proteinase 3. Compared to aprotinin, these variants have a net negative charge and are considered to have a reduced risk of kidney damage when administered to patients in large doses. In contrast, aprotinin has a nephrotoxic effect when administered in relatively high doses (Bayer, *Trasylol, Inhibitor of proteinase*;
30 Glaser *et al*, In "*Verhandlungen der Deutschen Gesellschaft Für Innere Medizin, 78. Kongress*", Bergmann, München, 1972, pp 1612-1614). This nephrotoxicity

is considered to be a consequence of the strongly net positive charge of aprotinin that causes it to bind to the negatively charged surfaces of kidney tubuli.

While there is no doubt that the anti-fibrinolytic clinical use of aprotinin reduces blood loss during vascular surgery, there is evidence of increased incidence of 'rebound thrombosis' which manifests in graft occlusion and perioperative myocardial infarction (Van der Meer *et al*, 1996, *Thromb. Haemost.* 75:1-3; Cosgrove *et al*, 1992, *Annals Thorac. Surg.* 54:1031-1038; Samama *et al*, 1994, *Thromb. Haemost.* 71:663-669). Consistent with these findings, it has been shown that aprotinin has a somewhat broad specificity and slow tight-binding kinetic action on plasmin (Willmott *et al*, 1995, *supra*). Accordingly, the increased incidence of rebound thrombosis may be a consequence of the tight binding of aprotinin to plasmin and concomitant irreversible neutralisation of the fibrinolytic system.

Until recently, there were no effective anti-fibrinolytic agents described in the prior art with reduced propensity for causation of rebound thrombosis compared to aprotinin. However, in a recent study, Willmott *et al* (1995, *supra*) isolated and characterised a plasmin inhibitor from the venom of the Australian brown snake, *Pseudonaja textilis textilis* with a promising kinetic profile in respect of rebound thrombosis. This isolated preparation of plasmin inhibitor, termed Textilinin (Txln), was found to consist of a single approximately 7 kDa protein, as assessed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. In contrast to the many serine protease enzymes inhibited by aprotinin, Txln was only shown to inhibit plasmin and trypsin. It was also shown to conform to a single stage competitive reversible mechanism for the binding of plasmin. In contrast, aprotinin conforms to a two stage reversible mechanism wherein enzyme and virgin inhibitor react to initially produce a loose non-covalent complex followed by a tightly bound, stable complex in which enzyme and inhibitor remain largely unchanged (Laskowski and Kato, 1980, *Annu. Rev. Biochem.* 49:593-626; Travis and Salvesen, 1983, *Annu. Rev. Biochem.* 52:655-709; Longstaff and Gaffney, 1991, *Biochemistry* 30:979-986). Moreover, Txln was shown to bind plasmin more rapidly (dissociation rate constant, $k_1 = 3.85 \times 10^{-5} \text{ sec}^{-1} \text{ M}^{-1}$) and with a less avid K_i

(dissociation constant, $K_i = 1.4 \times 10^{-8}$ M) than aprotinin (dissociation rate constant, $k_2 = 1.64 \times 10^{-5} \text{ sec}^{-1} \text{ M}^{-1}$; dissociation constant, $K_i = 5.3 \times 10^{-11}$ M - this latter value being in close agreement with a previously reported value of $K_i = 2 \times 10^{-10}$ M (Longstaff and Gaffney, 1992, *Fibrinolysis* 3:89-87)). It was suggested therefore
5 that the Txln kinetic profile may be clinically more attractive with respect to rebound thrombosis than that of aprotinin in the management of perioperative and postoperative bleeding.

SUMMARY OF THE INVENTION

The present invention results from the unexpected discovery of two
10 different plasmin inhibitors in the plasmin inhibitor preparation of Willmott *et al* (1995, *supra*) which was considered initially to be substantially homogeneous. Surprisingly, these plasmin inhibitors, termed Textilinin 1 (Txln 1) and Textilinin 2 (Txln 2) co-migrate with a molecular mass of about 7 kDa, as assessed by SDS-PAGE, and constitute only about 50% of the total protein (by weight) in the
15 parent plasmin inhibitor preparation used by Willmott and colleagues. This, together with the fact that Txln 1 and Txln 2 each have a different kinetic profile compared to the parent preparation, suggests that the parent preparation contains other compounds which may interfere with plasmin inhibition. In particular, Txln 1 and Txln 2 have distinct amino acid sequences, somewhat similar kinetic
20 profiles (Txln 1, $k_1 = 3.09 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$; $K_i = 3.5 \times 10^{-9}$ M; Txln 2, $k_1 = 8.20 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$; $K_i = 2.0 \times 10^{-9}$ M), while both inhibit blood loss in a murine model. Like the parent counterpart, Txln 1 and Txln 2 react only with plasmin and trypsin and therefore have high enzyme specificity compared to aprotinin. Moreover, comparison of the respective kinetic profiles of Txln 1, Txln 2 and aprotinin for
25 plasmin reveals that Txln 1 and Txln 2 are between 10-fold and 100-fold less efficient than aprotinin in inhibiting plasmin. It has also been found that Txln1 and Txln 2 dissociate from plasmin between 10-fold and 100-fold more rapidly than aprotinin. Due to their high specificity for plasmin and low inhibitory efficiency, Txln 1 and Txln 2 may therefore have a therapeutic advantage,
30 compared to aprotinin, to transiently affect the delicate balance between enzymes and inhibitors of the fibrinolytic system controlling the fluidity of blood.

The inventors have also found surprisingly that the Australian brown snake not only expresses transcripts encoding Txln 1 and Txln 2, but expresses transcripts encoding four additional plasmin inhibitors designated Textilinin 3, 4, 5 and 6 (*ie.*, Txln 3, Txln 4, Txln 5 and Txln 6). Although these latter transcripts appear to be expressed at significantly lower levels compared to those encoding Txln 1 and Txln 2, they are highly homologous to Txln 1 and Txln 2 both at the nucleotide level and the deduced amino acid level.

Thus, in one aspect of the invention, there is provided a substantially pure preparation of a plasmin inhibitor characterised in that it is a single stage competitive inhibitor of plasmin.

Preferably, said single-stage competitive inhibitor has a dissociation constant for plasmin in the range of from $1 \times 10^{-8} \text{ M}^{-1}$ to $1 \times 10^{-10} \text{ M}^{-1}$, more preferably from $5 \times 10^{-8} \text{ M}^{-1}$ to $8 \times 10^{-9} \text{ M}^{-1}$, most preferably from $1 \times 10^{-9} \text{ M}^{-1}$ to $5 \times 10^{-9} \text{ M}^{-1}$.

The single-stage competitive inhibitor may have a dissociation rate constant for plasmin in the range of from $4 \times 10^{-5} \text{ sec}^{-1} \text{ M}^{-1}$ to $5 \times 10^{-7} \text{ sec}^{-1} \text{ M}^{-1}$, more preferably from $1 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$ to $1 \times 10^{-7} \text{ sec}^{-1} \text{ M}^{-1}$, most preferably from $2 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$ to $9 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$.

Suitably, the single-stage competitive inhibitor comprises a polypeptide. Preferably, the polypeptide is selected from the group consisting of:

- (a) Lys-Asp-Arg-Pro-Asp-Phe-Cys-Glu-Leu-Pro-Ala-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Lys-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:2];
- (b) Lys-Asp-Arg-Pro-Glu-Leu-Cys-Glu-Leu-Pro-Pro-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Gln-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:4];
- (c) Lys-Asp-Arg-Pro-Asn-Phe-Cys-Lys-Leu-Pro-Ala-Glu-Thr-Gly-Arg-Cys-Asn-Ala-Lys-Ile-Pro-Arg-Phe-Tyr-Tyr-Asn-Pro-Arg-Gln-His-Gln-Cys-Ile-Glu-

Phe-Leu-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Ala-Asn-Asn-Phe-Lys-Thr-Ile-Lys-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:6];

(d) Lys-Asp-His-Pro-Lys-Phe-Cys-Glu-Leu-Pro-Ala-Glu-Thr-Gly-Ser-Cys-Lys-Gly-Asn-Val-Pro-Arg-Phe-Tyr-Tyr-Asn-Ala-Asp-His-His-Gln-Cys-Leu-Lys-Phe-Ile-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Ala-Asn-Asn-Phe-Lys-Thr-Ile-Glu-Glu-Gly-Lys-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:8];

(e) Lys-Asp-Arg-Pro-Lys-Phe-Cys-Glu-Leu-Leu-Pro-Asp-Thr-Gly-Ser-Cys-Glu-Asp-Phe-Thr-Gly-Ala-Phe-His-Tyr-Ser-Thr-Arg-Asp-Arg-Glu-Cys-Ile-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:10]; and

(f) Lys-Asp-Arg-Pro-Lys-Phe-Cys-Glu-Leu-Pro-Ala-Asp-Ile-Gly-Pro-Trp-Asp-Asp-Phe-Thr-Gly-Ala-Phe-His-Tyr-Ser-Pro-Arg-Glu-His-Glu-Cys-Ile-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Lys-Gly-Asn-Ala-Asn-Asn-Phe-Asn-Thr-Gln-Glu-Gln-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:12];

(g) a biologically-active fragment of any one of SEQ ID NO:2, 4, 6, 8, 10 and 12; and

(h) a variant or derivative of any of the foregoing polypeptides or fragments thereof.

Preferably, the variant has the general formula:

KDZPZYCZLBBZBGXCZXXBXFÄYXBZZZZCBZFBYGGC

XBNANNFXTXEECESTCAA (I), wherein: -

X is any amino acid;

Y is a hydrophobic amino acid;

Ä is an aromatic amino acid;

Z is K, R, H, D, E, Q or N; and

B is a neutral amino acid, or P, A, G, S, T, V or L.

Preferably, the Z at position 3 is H or R.

Suitably, the Z at position 5 is K, N, E or D.

- Preferably, the \ddot{Y} at position 6 is F or L.
- The Z at position 8 may be E or K.
- Suitably, the B at position 10 is P or L.
- Preferably, the B at position 11 is P or A.
- 5 The Z at position 12 is preferably E or D.
- Suitably, the B at position 13 is T or I.
- The X at position 15 may be P, S or R.
- The Z at position 17 is suitably K, N, E, D or R.
- Preferably, the X at position 18 is D, G, A or V.
- 10 Suitably, the X at position 19 is F, N, K or R.
- The X at position 20 is preferably T, P, F or I.
- The B at position 21 may be G, V or P.
- Suitably, the X at position 22 is A, S or R.
- Preferably, the \tilde{A} at position 24 is Y or H.
- 15 The X at position 26 is suitably S or N.
- The B at position 27 is preferably P, A or T.
- The Z at position 28 may be D or R.
- Suitably, the Z at position 29 is E, D, H or Q.
- Preferably, the Z at position 30 is H, K, R or Q.
- 20 The Z at position 31 may be K, Q or E.
- The B at position 33 is preferably L or I.
- The Z at position 34 is suitably E or K.
- Suitably, the B at position 36 is L or I.
- Preferably, the X at position 41 is E, G or K.
- 25 The B at position 42 may be C, but is preferably G.

Suitably, the X at position 48 is K, N or I.

Preferably, the X at position 50 is K, Q or I.

The polypeptide may comprise a leader peptide. Suitably, the leader peptide comprises the sequence of amino acids:-

5 Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-Glu-Val-Leu-Thr-Pro-Val-Ser-Ser [SEQ ID NO:14] a biologically-active fragment thereof, or variant or derivative of these.

Exemplary polypeptides which include the leader peptide may be selected from the group consisting of:-

- 10 i. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-Arg-Pro-Asp-Phe-Cys-Glu-Leu-Pro-Ala-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Lys-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:16];
- 15 ii. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-Arg-Pro-Glu-Leu-Cys-Glu-Leu-Pro-Pro-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Gln-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:18];
- 20 iii. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-Arg-Pro-Asn-Phe-Cys-Lys-Leu-Pro-Ala-Glu-Thr-Gly-Arg-Cys-Asn-Ala-Lys-Ile-Pro-Arg-Phe-Tyr-Tyr-Asn-Pro-Arg-Gln-His-Gln-Cys-Ile-Glu-Phe-Leu-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Ala-Asn-Asn-Phe-Lys-Thr-Ile-Lys-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala [SEQ ID NO:20];
- 25 iv. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-His-Pro-Lys-Phe-Cys-Glu-Leu-Pro-Ala-Glu-Thr-Gly-Ser-Cys-Lys-Gly-Asn-Val-Pro-Arg-Phe-Tyr-Tyr-Asn-
- 30

Ala-Asp-His-His-Gln-Cys-Leu-Lys-Phe-Ile-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-
Ala-Asn-Asn-Phe-Lys-Thr-Ile-Glu-Glu-Gly-Lys-Ser-Thr-Cys-Ala-Ala [SEQ
ID NO:22];

- 5 v. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-
Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-Arg-Pro-Lys-Phe-Cys-Glu-Leu-
Leu-Pro-Asp-Thr-Gly-Ser-Cys-Glu-Asp-Phe-Thr-Gly-Ala-Phe-His-Tyr-Ser-
Thr-Arg-Asp-Arg-Glu-Cys-Ile-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-
Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Ala-Ala;
[SEQ ID NO:24]; and
- 10 vi. Met-Ser-Ser-Gly-Gly-Leu-Leu-Leu-Leu-Leu-Gly-Leu-Leu-Thr-Leu-Trp-
Glu-Val-Leu-Thr-Pro-Val-Ser-Ser-Lys-Asp-Arg-Pro-Lys-Phe-Cys-Glu-Leu-
Pro-Ala-Asp-Ile-Gly-Pro-Trp-Asp-Asp-Phe-Thr-Gly-Ala-Phe-His-Tyr-Ser-
Pro-Arg-Glu-His-Glu-Cys-Ile-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Lys-Gly-Asn-
Ala-Asn-Asn-Phe-Asn-Thr-Gln-Glu-Gln-Cys-Glu-Ser-Thr-Cys-Ala-Ala;
15 [SEQ ID NO:26].

According to another aspect, the invention provides an isolated
polynucleotide encoding a polypeptide or biologically active fragment thereof, or
variant or derivative of said fragment or polypeptide, according to the first-
mentioned aspect. Suitably, said polynucleotide is selected from the group
20 consisting of:

- (1) AAGGACCGTCCGGATTTCTGTGAACTGCCTGCTGACACCGGAC
CATGTAGAGTCAGATTCCCATCCTTCTACTACAACCCAGATGAA
AAAAAGTGCTAGAGTTTATTTATGGTGGATGCGAAGGGAATGC
TAACAATTTTATCACCAAAGAGGAATGCGAAAGCACCTGTGCT
25 GCCTGA [SEQ ID NO:1];
- (2) AAGGACCGTCCAGAGTTGTGTGAACTGCCTCCTGACACCGGAC
CATGTAGAGTCAGATTCCCATCCTTCTACTACAACCCAGATGAA
CAAAAATGCCTAGAGTTTATTTATGGTGGATGCGAAGGGAATG
CTAACAATTTTATCACCAAAGAGGAATGCGAAAGCACCTGTGC
30 TGCCTGA [SEQ ID NO:3];

- (3) AAGGACCGTCCAAATTTCTGTAACTGCCTGCTGAAACCGGAC
GATGTAATGCCAAAATCCCACGCTTCTACTACAACCCACGTCAA
CATCAATGCATAGAGTTTCTCTATGGTGGATGCGGAGGGAATG
CTAACAATTTTAAGACCATTAAGGAATGCGAAAGCACCTGTGC
5 TGCATGA [SEQ ID NO:5];
- (4) AAGGACCATCCAAAATTCTGTGAACTCCCTGCTGAAACCGGAT
CATGTAAAGGCAACGTCCCACGCTTCTACTACAACGCAGATCA
TCATCAATGCCTAAAATTTATTTATGGTGGATGTGGAGGGAATG
CTAACAATTTTAAGACCATAGAGGAAGGCAAAGCACCTGTGC
10 TGCCTGA [SEQ ID NO:7];
- (5) AAGGACCGTCCAAAATTCTGTGAACTGCTTCCTGACACCGGATC
ATGTGAAGACTTTACCGGAGCCTTCCACTACAGCACACGTGATC
GTGAATGCATAGAGTTTATTTATGGTGGATGCGGAGGGAATGC
TAACAATTTTATCACCAAAGAGGAATGCGAAAGCACCTGTGCT
15 GCCTGA [SEQ ID NO:9];
- (6) AAGGACCGTCCAAAGTTCTGTGAACTGCCTGCTGACATCGGAC
CATGGGATGACTTTACCGGAGCCTTCCACTACAGCCCACGTGA
ACATGAATGCATAGAGTTTATTTATGGTGGATGCAAAGGGAAT
GCTAACAACCTTTAATACCCAAGAGCAATGCGAAAGCACCTGTG
20 CTGCCTGA [SEQ ID NO:11];
- (7) a polynucleotide fragment of any one of SEQ ID NOS 1, 3, 5, 7, 9, and 11
which fragment encodes a biologically-active polypeptide fragment of any
one of SEQ ID NO:2, 4, 6, 8, 10 and 12; and
- (8) a polynucleotide homologue of any of the foregoing sequences.

25 The polynucleotide preferably comprises a nucleotide sequence
encoding a leader peptide. Suitably, said nucleotide sequence comprises the
sequence of nucleotides:-

ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCA
CCCTCTGGGAGGTGCTGACCCCCGTCTCCAGC [SEQ ID NO:13] or a
30 biologically active fragment thereof, or a polynucleotide homologue of these.

Exemplary polynucleotides comprising said nucleotide sequence may be selected from the group consisting of:

- 1) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
GGAGGTGCTGACCCCGTCTCCAGCAAGGACCGTCCGGATTCTGT
5 GAACTGCCTGCTGACACCGGACCATGTAGAGTCAGATTCCCATCCT
TCTACTACAACCCAGATGAAAAAAGTGCCTAGAGTTTATTTATGG
TGGATGCGAAGGGAATGCTAACAATTTTATCACCAAAGAGGAATG
CGAAAGCACCTGTGCTGCCTGA [SEQ ID NO:15];
- 2) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
10 GGAGGTGCTGACCCCGTCTCCAGCAAGGACCGTCCAGAGTTGTGT
GAACTGCCTCCTGACACCGGACCATGTAGAGTCAGATTCCCATCCT
TCTACTACAACCCAGATGAACAAAATGCCTAGAGTTTATTTATGG
TGGATGCGAAGGGAATGCTAACAATTTTATCACCAAAGAGGAATG
CGAAAGCACCTGTGCTGCCTGA [SEQ ID NO:17];
- 15 3) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
GGAGGTGCTGACCCCGTCTCCAGCAAGGACCGTCCAAATTTCTGT
AAACTGCCTGCTGAAACCGGACGATGTAATGCCAAAATCCCACGC
TTCTACTACAACCCACGTCAACATCAATGCATAGAGTTTCTCTATG
GTGGATGCGGAGGGAATGCTAACAATTTTAAGACCATTAAAGGAAT
20 GCGAAAGCACCTGTGCTGCATGA [SEQ ID NO:19];
- 4) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
GGAGGTGCTGACCCCGTCTCCAGCAAGGACCATCCAAAATTCTGT
GAACTCCCTGCTGAAACCGGATCATGTAAAGGCAACGTCCCACGC
TTCTACTACAACGCAGATCATCATCAATGCCTAAAATTTATTTATG
25 GTGGATGTGGAGGGAATGCTAACAATTTTAAGACCATAGAGGAAG
GCAAAAGCACCTGTGCTGCCTGA [SEQ ID NO:21];
- 5) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
GGAGGTGCTGACCCCGTCTCCAGCAAGGACCGTCCAAAATTCTGT
GAACTGCTTCTGACACCGGATCATGTGAAGACTTTACCGGAGCCT
30 TCCACTACAGCACACGTGATCGTGAATGCATAGAGTTTATTTATGG
TGGATGCGGAGGGAATGCTAACAATTTTATCACCAAAGAGGAATG

CGAAAGCACCTGTGCTGCCTGA [SEQ ID NO:23];

- 6) ATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTCCTCACCTCTG
GGAGGTGCTGACCCCCGTCTCCAGCAAGGACCGTCCAAAGTTCTGT
GAACTGCCTGCTGACATCGGACCATGGGATGACTTTACCGGAGCCT
5 TCCACTACAGCCCACGTGAACATGAATGCATAGAGTTTATTTATGG
TGGATGCAAAGGGAATGCTAACAACCTTTAATACCCAAGAGCAATG
CGAAAGCACCTGTGCTGCCTGA [SEQ ID NO:25]; and
- 7) GGAGCTTCATCATGTCTTCTGGAGGTCTTCTTCTCCTGCTGGGACTC
CTCACCTCTGGGAGGTGCTGACCCCCGTCTCCAGCAAGGACCGTC
10 CAGAGTTGTGTGAACTGCCTCCTGACACCGGACCATGTAGAGTCAG
ATCCCCATCCTTCTACTACAACCCAGATGAACAAAAATGCCTAGAG
TTTATTTATGGTGGATGCGAAGGGAATGCTAACCAATTTTATCACC
AAAGAGGAATGCGAAAGCACCTGTGCTGCCTGAATGAGGAGACCC
TCCTGGATTGGATCGACAGTTCCAACCTTGACCCAAAGACCCTGCTT
15 CTGCCCTGGACCACCCTGGACACCCTTCCCCCAAACCCACCCTGG
ACTAATTCCTTTTCTCTGCAATAAAGCTTTGGTTCCAGCT [SEQ ID
NO:43]

In yet another aspect, the invention provides a pharmaceutical composition for alleviating blood loss in a patient, said composition comprising a polypeptide or a biological fragment thereof, or a variant or derivatives of these ("therapeutic agents") and a pharmaceutically acceptable carrier.

According to yet another aspect of the invention, there is provided a method for alleviating blood loss comprising the step of administering to a patient in need of such treatment a therapeutically effective dosage of a therapeutic agent of the invention in combination with a pharmaceutically acceptable carrier.

In a still further aspect, the invention resides in an anti-tumour agent comprising a polypeptide, polypeptide fragment, variant or derivative according to the invention conjugated with an anti-fibrin antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying drawings in which:

5 FIG. 1 shows a Sephacryl™ S-300 elution profile of venom from Australian brown snake. Five protein peaks (1-5) were obtained and plasmin inhibitory activity (e.g. Txln) was obtained on the shoulder peak 4 which comprises about 2% of the total protein applied to the column.

10 FIG. 2 depicts a DEAE-Sepharose™ CL-6B column elution profile of concentrated plasmin inhibitor activity derived from the Sephacryl™ S-300 chromatography in FIG. 1. The solid bars show two separate peaks of plasmin inhibitory activity (denoted 1 and 2).

15 FIG. 3 shows a Sephacryl™ S-100 elution profile of one of the two pooled and concentrated fractions obtained from the DEAE-Sepharose™ CL-6B chromatography. The profile shown is that of Txln 1 but the profile of Txln 2 is identical. Insert, however shows two distinct elution profiles for each of Txln 1 and Txln 2 using reverse-phase C 18 HPLC chromatography.

20 FIG. 4 illustrates a real time curve fit analysis using Sigmaplot of Txln 1 (0- 410 nM) inhibition of plasmin (2 nM). Similar inhibition curves (data not shown) were obtained with Txln 2.

22 FIG. 5 shows the amino acid sequences for Txln 1 and Txln 2, as well as those of Taicotoxin associated plasmin inhibitor (TAC) and aprotinin (APRO). The sequences were aligned according to the location of the six cysteines.

25 FIG. 6 lists a partial cDNA sequence of *Txln 1*. The amino acid sequence encoded by this partial sequence is shown below the nucleotide sequence in single letter code. The letter "N" denotes a non-characterized nucleotide.

30 FIG. 7 lists a partial cDNA sequence of *Txln 2*. The amino acid sequence encoded by this partial sequence is shown below the nucleotide

sequence in single letter code. The letter "N" denotes a non-characterized nucleotide.

FIG. 8 shows the electrophoretic mobility patterns on a 2% agarose gel stained with EtBr of PCR products obtained with *Txln* gene-specific primers: Lane 1, control (template, no primers); Lane 2, 5'-RACE PCR product; Lane 3, 3'-RACE PCR product; Lane M; size markers.

FIG. 9 lists the *Txln 1* cDNA sequence derived from nucleotide sequence analysis of the 5' and 3' RACE products.

FIG. 10 shows the nucleotide and deduced amino acid sequences relating to respective proforms of *Txln* 1-6.

FIG. 11 shows a sequence comparison of Textilinin polypeptide sequences using the PILEUP program of the GCG Wisconsin Suite.

FIG. 12 refers to a 15% SDS polyacrylamide gel electrophoresis under reducing conditions of Textilinin-GST fusion proteins expressed from various colonies harbouring pGEM-2T-*Txln 1* recombinant clones. Colonies were selected by PCR screening using sequence-specific primers. Numerals denote clone designation number.

DETAILED DESCRIPTION

1. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described.

For the purposes of the present invention, the following terms are defined below.

By "biologically-active fragment" means a fragment of a substantially full-length parent polypeptide wherein the fragment retains the activity of the parent polypeptide. For example, in the case of a biologically active fragment of a polypeptide according to SEQ ID NO:2, 4, 6, 8, 10 and 12, the polypeptide fragment must retain the single stage competitive inhibition

properties of the parent polypeptide with respect to plasmin.

The term "*biological sample*" as used herein refers to a sample that may be untreated, treated, diluted or concentrated from a patient. Suitably, the biological sample is selected from foetal cells, and tissue samples including
5 tissue from the caudate and/or putamen regions of the brain, and the like.

By "*corresponds to*" or "*corresponding to*" is meant (a) a polynucleotide having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or encoding an amino acid sequence identical to an amino acid sequence in a peptide
10 or protein; or (b) a peptide or polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference peptide or protein.

By "*derivative*" is meant a polypeptide that has been derived from the basic sequence by modification, for example by conjugation or complexing
15 with other chemical moieties or by post-translational modification techniques as would be understood in the art. The term "*derivative*" also includes within its scope alterations that have been made to a parent sequence including additions, or deletions that provide for functional equivalent molecules.

"*Homology*" refers to the percentage number of amino acids that
20 are identical or constitute conservative substitutions as defined in Table 1 below. Homology may be determined using sequence comparison programs such as GAP (Deveraux *et al.* 1984, *Nucleic Acids Research* 12, 387-395) which is incorporated herein by reference. In this way sequences of a similar or substantially different length to those cited herein might be compared by insertion
25 of gaps into the alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

"*Hybridisation*" is used herein to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related
30 by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA U pairs with A and C pairs with G. In this regard, the terms "match" and

“mismatch” as used herein refer to the hybridisation potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridize efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides that do not hybridise efficiently.

By “*isolated*” is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an “isolated polynucleotide”, as used herein, refers to a polynucleotide, which has been purified from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment.

By “*obtained from*” is meant that a sample such as, for example, a nucleic acid extract is isolated from, or derived from, a particular source of the host. For example, the nucleic acid extract may be obtained from tissue isolated directly from the host.

The term “*oligonucleotide*” as used herein refers to a polymer composed of a multiplicity of nucleotide units (deoxyribonucleotides or ribonucleotides, or related structural variants or synthetic analogues thereof) linked via phosphodiester bonds (or related structural variants or synthetic analogues thereof). Thus, while the term “oligonucleotide” typically refers to a nucleotide polymer in which the nucleotides and linkages between them are naturally occurring, it will be understood that the term also includes within its scope various analogues including, but not restricted to, peptide nucleic acids (PNAs), phosphoramidates, phosphorothioates, methyl phosphonates, 2-O-methyl ribonucleic acids, and the like. The exact size of the molecule may vary depending on the particular application. An oligonucleotide is typically rather short in length, generally from about 10 to 30 nucleotides, but the term can refer to molecules of any length, although the term “polynucleotide” or “nucleic acid” is typically used for large oligonucleotides.

By “*operably linked*” is meant that transcriptional and translational regulatory nucleic acids are positioned relative to a nucleotide sequence encoding

a polypeptide or fragment thereof in such a manner that transcription of said nucleotide sequence is initiatable and terminatable, respectively.

The term "*patient*" refers to patients of human or other animal origin and includes any individual it is desired to examine or treat using the methods of the invention. However, it will be understood that "*patient*" does not
5 imply that symptoms are present.

The term "*polynucleotide*" or "*nucleic acid*" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotides in length.

By "*pharmaceutically-acceptable carrier*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration.
10

The term "*polynucleotide homologues*" generally refers to polynucleotides that hybridise with a reference polynucleotide under substantially stringent conditions.
15

"*Polypeptide*", "*peptide*" and "*protein*" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers.
20

By "*primer*" is meant an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerising agent. The primer is preferably single-stranded for maximum efficiency in amplification but may alternatively be double-stranded. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerisation agent. The length of the primer depends on many factors, including application, temperature to be employed, template reaction conditions, other reagents, and source of primers.
25
30 For example, depending on the complexity of the target sequence, the

oligonucleotide primer typically contains 15 to 35 or more nucleotides, although it may contain fewer nucleotides. Primers can be large polynucleotides, such as from about 200 nucleotides to several kilobases or more. Primers may be selected to be "substantially complementary" to the sequence on the template to which it is designed to hybridise and serve as a site for the initiation of synthesis. By "substantially complementary", it is meant that the primer is sufficiently complementary to hybridize with a target nucleotide sequence. Preferably, the primer contains no mismatches with the template to which it is designed to hybridize but this is not essential. For example, non-complementary nucleotides may be attached to the 5'-end of the primer, with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary nucleotides or a stretch of non-complementary nucleotides can be interspersed into a primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize therewith and thereby form a template for synthesis of the extension product of the primer.

"Probe" refers to a molecule that binds to a specific sequence or sub-sequence or other moiety of another molecule. Unless otherwise indicated, the term "probe" typically refers to an oligonucleotide probe that binds to another nucleic acid, often called the "target nucleic acid", through complementary base pairing. Probes may bind target nucleic acids lacking complete sequence complementarity with the probe, depending on the stringency of the hybridisation conditions. Probes can be directly or indirectly labelled.

The term "*recombinant polynucleotide*" as used herein refers to a polynucleotide formed *in vitro* by the manipulation of nucleic acid into a form not normally found in nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the a nucleotide sequence.

By "*recombinant polypeptide*" is meant a polypeptide made using recombinant techniques, *i.e.*, through the expression of a recombinant polynucleotide.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". A "*reference sequence*" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "*comparison window*" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr. Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected.

"*Sequence identity*" refers to sequences that are identical (*i.e.*, on a nucleotide-by-nucleotide or amino acid-by-amino acid basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence similarity.

"*Stringency*" as used herein, refers to the temperature and ionic

strength conditions, and presence or absence of certain organic solvents, during hybridisation. The higher the stringency, the higher will be the degree of complementarity between immobilised nucleotide sequences and the labelled polynucleotide sequence.

5 "*Stringent conditions*" refers to temperature and ionic conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridize. The stringency required is nucleotide sequence dependent and also depends upon the various components present during hybridisation. Generally, stringent conditions are selected to be about 10
10 to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a target sequence hybridises to a complementary probe.

 The term "*substantially pure*" as used herein describes a
15 compound, *eg.*, a peptide which has been separated from components that naturally accompany it. Typically, a compound is substantially pure when at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity
20 can be measured by any appropriate method, *eg.*, in the case of peptides by chromatography, gel electrophoresis or HPLC analysis. A compound, *eg.*, a peptide is also substantially purified when it is essentially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state.

25 The term "*variant*" refers to polypeptides in which one or more amino acids have been replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions).

30 By "*vector*" is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into

which a synthetic nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integratable with the genome of the defined host such that the cloned sequence is reproducible. Thus, by "*expression vector*" is meant any autonomous element capable of directing the synthesis of a protein. Such expression vectors are well known by practitioners in the art. The vector may also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are well known to those of skill in the art.

As used herein, underscoring or italicising the name of a gene shall indicate the gene, in contrast to its protein product, which is indicated by the name of the gene in the absence of any underscoring or italicising. For example, "*Txln I*" shall mean the *Txln I* gene, whereas "Txln I" shall indicate the protein product of the "*Txln I*" gene.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

2. Plasmin inhibitors of the invention

The present invention provides a substantially pure preparation of a plasmin inhibitor characterised in that it is a single stage competitive inhibitor of plasmin. In a preferred embodiment, the single-stage competitive inhibitor has dissociation constant for plasmin in the range of from $1 \times 10^{-8} \text{ M}^{-1}$ to $1 \times 10^{-10} \text{ M}^{-1}$, more preferably from $5 \times 10^{-8} \text{ M}^{-1}$ to $8 \times 10^{-9} \text{ M}^{-1}$, most preferably from $1 \times 10^{-9} \text{ M}^{-1}$ to $5 \times 10^{-9} \text{ M}^{-1}$. The single-stage competitive inhibitor preferably has a dissociation rate constant for plasmin in the range of from $4 \times 10^{-5} \text{ sec}^{-1} \text{ M}^{-1}$ to $5 \times 10^{-7} \text{ sec}^{-1} \text{ M}^{-1}$, more preferably from $1 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$ to $1 \times 10^{-7} \text{ sec}^{-1} \text{ M}^{-1}$, and most preferably from $2 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$ to $9 \times 10^{-6} \text{ sec}^{-1} \text{ M}^{-1}$.

2.1 Textilinin Polypeptides²²

B

2.1. Textilinin Polypeptides

A The plasmin inhibitor is preferably a Textilinin polypeptide. Accordingly, the present invention provides an isolated polypeptide according to SEQ ID NOS 2, 4, 6, 8, 10, and 12, or biologically active fragment respectively thereof, or variant or derivative of these. SEQ ID NO:2 and SEQ ID NO:4 correspond respectively to the novel about 7 kDa Textilinin 1 (Txln 1) and Textilinin 2 (Txln 2) polypeptides obtained from *Pseudonaja textilis textilis*, as described more fully hereinafter. SEQ ID NOS 6, 8, 10 and 12 correspond to homologous polypeptides deduced from polynucleotides obtained from *Pseudonaja textilis textilis*.

In one embodiment, the isolated polypeptide may comprise a leader peptide according to SEQ ID NO:14 or biologically active fragment thereof, or variant or derivative of these. In this regard, the invention also provides an isolated polypeptide according to SEQ ID NO:16, 18, 20, 22, 24 and 26.

15

2.2 Textilinin Polypeptide fragments

B

2.2. Textilinin Polypeptide fragments

A The invention contemplates biologically active fragments of a Textilinin polypeptide according to the invention. Exemplary fragments of this type include deletion mutants and small peptides, for example of at least 15, preferably at least 20 and more preferably at least 30 contiguous amino acids of a polypeptide according to SEQ ID NO:2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24 and 26, which fragment consists retains single stage competitive inhibition of plasmin.

20

2.3 Textilinin Polypeptide variants

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2.3. Textilinin Polypeptide variants

A With regard to variant polypeptides of the invention, it will be understood that such variants should retain single stage competitive inhibition of plasmin of the parent or reference polypeptide. Exemplary conservative substitutions in a parent polypeptide may be made according to Table 1:

25

TABLE 1

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile,
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function are made by selecting substitutions that are less conservative than those shown in TABLE 1. Other replacements would be non-conservative substitutions and relatively fewer of these may be tolerated. Generally, the substitutions which are likely to produce the greatest changes in a polypeptide's properties are those in which: (a) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.*, Ala, Leu, Ile, Phe or Val); (b) a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an electronegative residue (*e.g.*, Glu or Asp); or (d) a residue having a bulky side chain (*e.g.*, Phe or Trp) is substituted for, or by, one

having a smaller side chain (e.g., Ala, Ser) or no side chain (e.g., Gly).

In general, variants comprise regions that are at least 75% homologous, more suitably at least 80%, preferably at least 85%, and most preferably at least 90% homologous to the basic sequences as for example shown in SEQ ID NO: 2, 4, 6, 8, 10 and 12. In an alternate embodiment, variants comprise regions that have at least 70%, more suitably at least 80%, preferably at least 90%, and most preferably at least 95% identity over a parent amino acid sequence of identical size ("*comparison window*") or when compared to an aligned sequence in which the alignment is performed by a computer homology program known in the art. What constitutes suitable variants may be determined by conventional techniques. For example, nucleic acids encoding polypeptides according to SEQ ID NO: 2, 4, 6, 8, 10 and 12 can be mutated using either random mutagenesis for example using transposon mutagenesis, or site-directed mutagenesis. The resultant DNA fragments are then cloned into suitable expression hosts such as *E. coli* using conventional technology and clones that retain the desired activity are detected. As mentioned above, the desired activity will include single stage competitive inhibition of plasmin of the parent or reference polypeptide. Where the clones have been derived using random mutagenesis techniques, positive clones would have to be sequenced in order to detect the mutation. The term "*variant*" also includes naturally occurring allelic variants.

In a preferred embodiment, the variant has the general formula:

KDZPZYCZLBBZBGXCZXXXBXFÄYXBZZZCBZFBYGGC

XBNANNFXTXEECESTCAA (I), wherein: -

25

- X is any amino acid;
- Y is a hydrophobic amino acid;
- Ä is an aromatic amino acid;
- Z is K, R, H, D, E, Q or N; and
- B is a neutral amino acid, or P, A, G, S, T, V or L.

2.4 Textilinin Polypeptide derivatives

B

2.4. Textilinin Polypeptide derivatives

A With reference to suitable derivatives of the invention, such derivatives include amino acid deletions and/or additions to a Textilinin polypeptide according to the invention such as, for example, SEQ ID NO:2, 4, 6, 8, 10 and 12, or variants thereof, wherein said derivatives retain single stage competitive inhibition of plasmin. "Additions" of amino acids may include fusion of the polypeptides, fragments thereof or variants of these with other polypeptides or proteins. In this regard, it will be appreciated that the polypeptides, polypeptide fragments or variants of the invention may be incorporated into larger polypeptides, and such larger polypeptides may also be expected to retain the single stage competitive inhibition of plasmin mentioned above.

The Textilinin polypeptides of the invention, fragments thereof or variants of these may be fused to a further protein, for example, which is not derived from the original host. The other protein may, by way of example, assist in the purification of the protein. For instance a polyhistidine tag, or a maltose binding protein may be used in this respect as described in more detail below. Alternatively, it may produce an antigenic response or immunogenic response that is effective against the polypeptide or fragment thereof. Other possible fusion proteins are those which produce an immunomodulatory response. Particular examples of such proteins include Protein A or glutathione S-transferase (GST).

Other derivatives contemplated by the invention include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the polypeptides, fragments and variants of the invention.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH₄; reductive alkylation by reaction with an aldehyde followed by reduction with NaBH₄; and

trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

5 The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivitization, by way of example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

10 Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides or by oxidation with N-bromosuccinimide.

20 Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

25 Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminoheptanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated by the present invention is shown in TABLE 2.

30

TABLE 2

Non-conventional amino acid	Non-conventional amino acid
α -aminobutyric acid	L-N-methylalanine
α -amino- α -methylbutyrate	L-N-methylarginine
aminocyclopropane-carboxylate	L-N-methylasparagine
aminoisobutyric acid	L-N-methylaspartic acid
aminonorbonyl-carboxylate	L-N-methylcysteine
cyclohexylalanine	L-N-methylglutamine
cyclopentylalanine	L-N-methylglutamic acid
L-N-methylisoleucine	L-N-methylhistidine
D-alanine	L-N-methylleucine
D-arginine	L-N-methyllysine
D-aspartic acid	L-N-methylmethionine
D-cysteine	L-N-methylnorleucine
D-glutamate	L-N-methylnorvaline
D-glutamic acid	L-N-methylornithine
D-histidine	L-N-methylphenylalanine
D-isoleucine	L-N-methylproline
D-leucine	L-N-methylserine
D-lysine	L-N-methylthreonine
D-methionine	L-N-methyltryptophan
D-ornithine	L-N-methyltyrosine
D-phenylalanine	L-N-methylvaline
D-proline	L-N-methylethylglycine
D-serine	L-N-methyl-t-butylglycine
D-threonine	L-norleucine
D-tryptophan	L-norvaline
D-tyrosine	α -methyl-aminoisobutyrate
D-valine	α -methyl- γ -aminobutyrate
D- α -methylalanine	α -methylcyclohexylalanine
D- α -methylarginine	α -methylcyclopentylalanine

D- α -methyloasparagine	α -methyl- α -naphthylalanine
D- α -methyloaspartate	α -methylpenicillamine
D- α -methylcysteine	N-(4-aminobutyl)glycine
D- α -methylglutamine	N-(2-aminoethyl)glycine
D- α -methylhistidine	N-(3-aminopropyl)glycine
D- α -methylisoleucine	N-amino- α -methylbutyrate
D- α -methylleucine	α -naphthylalanine
D- α -methyllysine	N-benzylglycine
D- α -methylmethionine	N-(2-carbamylethyl)glycine
D- α -methylornithine	N-(carbamylmethyl)glycine
D- α -methylphenylalanine	N-(2-carboxyethyl)glycine
D- α -methylproline	N-(carboxymethyl)glycine
D- α -methylserine	N-cyclobutylglycine
D- α -methylthreonine	N-cycloheptylglycine
D- α -methyltryptophan	N-cyclohexylglycine
D- α -methyltyrosine	N-cyclodecylglycine
L- α -methylleucine	L- α -methyllysine
L- α -methylmethionine	L- α -methylnorleucine
L- α -methylnorvaline	L- α -methylornithine
L- α -methylphenylalanine	L- α -methylproline
L- α -methylserine	L- α -methylthreonine
L- α -methyltryptophan	L- α -methyltyrosine
L- α -methylvaline	L-N-methylhomophenylalanine
N-(N-(2,2-diphenylethyl carbamylmethyl)glycine	N-(N-(3,3-diphenylpropyl carbamylmethyl)glycine
1-carboxy-1-(2,2-diphenyl-ethyl amino)cyclopropane	

The invention also contemplates the use of crosslinkers, for example, to stabilise 3D conformations of the peptides or peptide homologs of the invention, using homo-bifunctional cross linkers such as bifunctional imido esters

having $(\text{CH}_2)_n$ spacer groups with $n = 1$ to $n = 6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety or carbodiimide. In addition, peptides can be conformationally constrained, for example, by introduction of double bonds between C_α and C_β atoms of amino acids, by incorporation of C_α and N_α -methylamino acids, and by formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini between two side chains or between a side chain and the N or C terminus of the peptides or analogues. For example, reference may be made to: Marlowe (1993, *Biorganic & Medicinal Chemistry Letters* 3:437-44, hereby incorporated by reference) which describes peptide cyclization on TFA resin using trimethylsilyl (TMSE) ester as an orthogonal protecting group; Pallin and Tam (1995, *J. Chem. Soc. Chem. Comm.* 2021-2022, hereby incorporated by reference) which describes the cyclization of unprotected peptides in aqueous solution by oxime formation; Algin *et al* (1994, *Tetrahedron Letters* 35: 9633-9636, hereby incorporated by reference) which discloses solid-phase synthesis of head-to-tail cyclic peptides *via* lysine side-chain anchoring; Kates *et al* (1993, *Tetrahedron Letters* 34: 1549-1552, hereby incorporated by reference) which describes the production of head-to-tail cyclic peptides by three-dimensional solid phase strategy; Tumelty *et al* (1994, *J. Chem. Soc. Chem. Comm.* 1067-1068, hereby incorporated by reference) which describes the synthesis of cyclic peptides from an immobilized activated intermediate, wherein activation of the immobilized peptide is carried out with *N*-protecting group intact and subsequent removal leading to cyclization; McMurray *et al* (1994, *Peptide Research* 7:195-206, hereby incorporated by reference) which discloses head-to-tail cyclization of peptides attached to insoluble supports by means of the side chains of aspartic and glutamic acid; Hruby *et al* (1994, *Reactive Polymers* 22:231-241, hereby incorporated by reference) which teaches an alternate method for cyclizing peptides *via* solid supports; and Schmidt and Langer (1997, *J. Peptide Res.* 49:67-73, hereby incorporated by reference) which discloses a method for synthesizing cyclotetrapeptides and cyclopentapeptides. The

foregoing methods may be used to produce conformationally constrained peptides with single stage competitive inhibition kinetics in respect of plasmin.

The invention also contemplates Textilinin polypeptides or biologically active fragments thereof that have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent.

The present invention further encompasses chemical analogues of Textilinin polypeptides or biologically active fragments thereof, which analogues act as functional analogues of said polypeptides or fragments. In this regard, chemical analogues may not necessarily be derived from said polypeptides or fragments but may share certain conformational similarities. Alternatively, chemical analogues may be specifically designed to mimic certain physical properties of said polypeptides or fragments. Chemical analogues may be chemically synthesized or may be detected following, for example, natural product screening.

Textilinin polypeptides may be prepared by any suitable procedure known to those of skill in the art. For example, such polypeptides may be prepared by a procedure including the steps of:

- (a) preparing a recombinant polynucleotide containing a nucleotide sequence encoding a Textilinin polypeptide, for example, SEQ ID NO:2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24 or 26, or biologically active fragment respectively thereof, or variant or derivative of these, which nucleotide sequence is operably linked to transcriptional and translational regulatory nucleic acid;
- (b) introducing into a suitable host cell the recombinant polynucleotide;
- (c) culturing the host cell to express recombinant polypeptide from said recombinant polynucleotide; and
- (d) isolating the recombinant polypeptide.

Suitably, said recombinant polynucleotide comprises an isolated

natural Textilinin sequence. For example, such polynucleotide may be selected from any one of SEQ ID NO:1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 25 or 43.

5 The recombinant polynucleotide preferably comprises an expression vector that may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome.

The transcriptional and translational regulatory nucleic acid will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells.

10 Typically, the transcriptional and translational regulatory nucleic acid may include, but is not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

15 Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter.

20 In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

25 The expression vector may also include a fusion partner (typically provided by the expression vector) so that the recombinant polypeptide of the invention is expressed as a fusion polypeptide with said fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of said fusion polypeptide.

In order to express said fusion polypeptide, it is necessary to ligate a nucleotide sequence according to the invention into the expression vector so that the translational reading frames of the fusion partner and the nucleotide sequence of the invention coincide.

30 Well known examples of fusion partners include, but are not

limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS₆), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices
5 for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS₆) fusion partners and the Pharmacia GST purification system.

Another fusion partner well known in the art is green fluorescent
10 protein (GFP). This fusion partner serves as a fluorescent "tag" which allows the fusion polypeptide of the invention to be identified by fluorescence microscopy or by flow cytometry. The GFP tag is useful when assessing subcellular localization of the fusion polypeptide of the invention, or for isolating cells which express the fusion polypeptide of the invention. Flow cytometric methods such as
15 fluorescence activated cell sorting (FACS) are particularly useful in this latter application.

Preferably, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the
20 recombinant polypeptide of the invention therefrom. The liberated polypeptide can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "epitope tags", which are usually short peptide sequences for which a
25 specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-Myc, influenza virus, haemagglutinin and FLAG tags.

The step of introducing into the host cell the recombinant polynucleotide may be effected by any suitable method including transfection,
30 and transformation, the choice of which will be dependent on the host cell employed. Such methods are well known to those of skill in the art.

Recombinant polypeptides of the invention may be produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a Textilinin polypeptide, fragment, variant or derivative according to the invention. The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained by one skilled in the art through routine experimentation.

Suitable host cells for expression may be prokaryotic or eukaryotic. One preferred host cell for expression of a polypeptide according to the invention is a bacterium. The bacterium used may be *Escherichia coli*. Alternatively, the host cell may be an insect cell such as, for example, *SF9* cells that may be utilised with a baculovirus expression system.

The recombinant protein may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook, *et al.*, MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbor Press, 1989), incorporated herein by reference, in particular Sections 16 and 17; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons, Inc. 1994-1998), incorporated herein by reference, in particular Chapters 10 and 16; and Coligan *et al.*, CURRENT PROTOCOLS IN PROTEIN SCIENCE (John Wiley & Sons, Inc. 1995-1997) which is incorporated by reference herein, in particular Chapters 1, 5 and 6.

In some cases, the recombinant polypeptide may require refolding. Exemplary methods of refolding polypeptides include those as for example described by Bieri *et al.* (1995, *Biochemistry*, 34:13059-13065) and Norris *et al.* (1994, US. Patent 5,373,090 to Novo Nordisk), which are incorporated herein by reference.

Alternatively, the Textilinin polypeptides, polypeptide fragments, or variants or derivatives of these, may be synthesized using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications.

3. Polynucleotides of the invention

3.1. Textilinin polynucleotides

The invention further provides a polynucleotide that encodes a Textilinin polypeptide, fragment, variant or derivative as defined above. Suitably said polynucleotide is selected from the group consisting of:- SEQ ID NO: 1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 25 and 43; a polynucleotide fragment of any one of SEQ ID NO: 1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 25 or 43; and a polynucleotide homologue of the foregoing sequences. Preferably, these sequences encode a product displaying single stage competitive inhibition of plasmin as defined above.

As will be more fully described hereinafter, a family of *Textilinin* (*Txln*) genes encoding single stage competitive inhibitors of plasmin has been obtained from *Pseudonaja textilis textilis*. SEQ ID NO:1 corresponds to a portion of the *Txln 1* gene that encodes the mature Txln 1 polypeptide of about 7 kDa as defined in SEQ ID NO:2. SEQ ID NO:3 corresponds to a portion of the *Txln 2* gene that encodes the mature Txln 2 polypeptide of about 7 kDa as defined in SEQ ID NO:4. SEQ ID NO:5, 7, 9 and 11, correspond respectively to portions of the *Txln 3*, *Txln 4*, *Txln 5* and *Txln 6* genes. These portions encode mature Txln 3, 4, 5 and 6 polypeptides, respectively.

The invention also provides full-length open reading frame (ORF) polynucleotides in relation to *Txln 1*, *Txln 2*, *Txln 3*, *Txln 4*, *Txln 5* and *Txln 6*. Each said full-length polynucleotide comprises a first sequence encoding a 24-residue leader peptide, and a second sequence encoding a mature Txln polypeptide. The first sequence preferably comprises SEQ ID NO:15. SEQ ID NO:17, 19, 21, 23, and 25 correspond respectively to full-length ORF polynucleotides for *Txln 1*, *Txln 2*, *Txln 3*, *Txln 4*, *Txln 5* and *Txln 6*. SEQ ID NO:43 corresponds to the largest cDNA sequence obtained for *Txln 1*, comprising 5' UTR and a 3'UTR sequences in addition to the ORF sequence.

Alternatively, a polynucleotide sequence encoding the Textilinin polypeptides or polypeptide fragments of the invention may be conveniently prepared by taking advantage of the genetic code and synthesising, for example,

by use of an oligonucleotide sequencer, a sequence of nucleotides which when translated by a host cell results in the production of a polypeptide according to SEQ ID NO:2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24 or 26, polypeptide fragments thereof.

3.2 Polynucleotide homologues

5 B

3.2. Polynucleotide homologues

A Suitable polynucleotide homologues of the invention may be prepared according to the following procedure:

- (i) obtaining a nucleic acid extract from a suitable host;
- (ii) creating primers which are optionally degenerate wherein each comprises a portion of a reference polynucleotide; and
- (iii) using said primers to amplify, via nucleic acid amplification techniques, at least one amplification product from said nucleic acid extract, wherein said amplification product corresponds to a polynucleotide homologue.

10

15

The host from which a nucleic acid extract is obtained is preferably a snake. Suitable snakes may be selected from the group consisting of the family *Elapidae*, and the family *Viperidae*.

Suitably, the primers are selected from the group consisting of:-

- (A) ATGAARGAYAGRCCHGARYTNGAR [SEQ ID NO:27];
- (B) GTRCTYTCRTGYTCYTCY [SEQ ID NO:28];
- (C) ATATATGGATCCAAGGACCGGCCTGACTTC [SEQ ID NO:29];
- (D) AACGGGAATTCTCAGAGCCACACGTGCTTTC [SEQ ID NO:30];
- (E) AACGGGAATTCTCATGAGCCACAGGTAGACTC [SEQ ID NO:31];
- (F) CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAG
T [SEQ ID NO:32];
- (G) CTAATACGACTCACTATAGGGC [SEQ ID NO:33];
- (H) AAGCAGTGGTAACAACGCAGAGT [SEQ ID NO:34];

25

- (I) ATCAGCGGATCCATGTCTGGAGGT [SEQ ID NO:35];
(J) TCTCCTGAATTCTCAGGCAGCACAGGT [SEQ ID NO:36];
(K) ATTATAGGATCCAAGGACCGTCCGGAT [SEQ ID NO:37];
(L) ATTATAGGATCCAAGGACCGTCCAGAG [SEQ ID NO:38];
5 (M) AACGTCGGATCCAAGGACCGTCCAAAT [SEQ ID NO:39];
(N) AACGTCGGATCCAAGGACCATCCAAAA [SEQ ID NO:40];
(O) AACGTCGGAT TCAAGGACCG TCCAAAA [SEQ ID NO:41];
(P) ATTGTCGGATCCAAGGACCTGCCAAAG [SEQ ID NO:42].

Alternatively, a polynucleotide homologue of the invention may be
10 obtained from a polynucleotide library derived from a tissue of a snake. Such a
library may be a snake cDNA library or snake genomic DNA library.

Suitable nucleic acid amplification techniques are well known to
the skilled addressee, and include polymerase chain reaction (PCR) as for
example described in Ausubel *et al.* (1994-1998, *supra*, Chapter 15) which is
15 incorporated herein by reference; strand displacement amplification (SDA) as for
example described in U.S. Patent No 5,422,252 which is incorporated herein by
reference; rolling circle replication (RCR) as for example described in Liu *et al.*,
(1996, *J. Am. Chem. Soc.* 118:1587-1594 and International application WO
92/01813) and Lizardi *et al.*, (International Application WO 97/19193) which are
20 incorporated herein by reference; nucleic acid sequence-based amplification
(NASBA) as for example described by Sooknanan *et al.*, (1994, *Biotechniques*
17:1077-1080) which is incorporated herein by reference; and Q- β replicase
amplification as for example described by Tyagi *et al.*, (1996, *Proc. Natl. Acad.*
Sci. USA 93:5395-5400) which is incorporated herein by reference.

25 Typically, polynucleotide homologues that are substantially
complementary to a reference polynucleotide are identified by blotting techniques
that include a step whereby nucleic acids are immobilized on a matrix (preferably
a synthetic membrane such as nitrocellulose), followed by a hybridisation step,
and a detection step. Southern blotting is used to identify a complementary DNA

sequence; northern blotting is used to identify a complementary RNA sequence. Dot blotting and slot blotting can be used to identify complementary DNA/DNA, DNA/RNA or RNA/RNA polynucleotide sequences. Such techniques are well known by those skilled in the art, and have been described in Ausubel *et al.* (1994-1998, *supra*) at pages 2.9.1 through 2.9.20.

According to such methods, Southern blotting involves separating DNA molecules according to size by gel electrophoresis, transferring the size-separated DNA to a synthetic membrane, and hybridising the membrane-bound DNA to a complementary nucleotide sequence labelled radioactively, enzymatically or fluorochromatically. In dot blotting and slot blotting, DNA samples are directly applied to a synthetic membrane prior to hybridisation as above.

An alternative blotting step is used when identifying complementary polynucleotides in a cDNA or genomic DNA library, such as through the process of plaque or colony hybridisation. A typical example of this procedure is described in Sambrook *et al.*, (1989, *supra*) Chapters 8-12.

Typically, the following general procedure can be used to determine hybridisation conditions. Polynucleotides are blotted/transferred to a synthetic membrane, as described above. A reference polynucleotide such as a polynucleotide of the invention is labelled as described above, and the ability of this labelled polynucleotide to hybridise with an immobilised polynucleotide analyzed.

A skilled addressee will recognize that a number of factors influence hybridization. The specific activity of radioactively labelled polynucleotide sequence should typically be greater than or equal to about 10^8 dpm/mg to provide a detectable signal. A radiolabelled nucleotide sequence of specific activity 10^8 to 10^9 dpm/mg can detect approximately 0.5 pg of DNA. It is well known in the art that sufficient DNA must be immobilized on the membrane to permit detection. It is desirable to have excess immobilized DNA, usually 10 μ g. Adding an inert polymer such as 10% (w/v) dextran sulfate (MW 500,000) or polyethylene glycol 6000 during hybridization can also increase the sensitivity of

hybridization (see Ausubel *supra* at 2.10.10).

To achieve meaningful results from hybridisation between a polynucleotide immobilized on a membrane and a labelled polynucleotide, a sufficient amount of the labelled polynucleotide must be hybridised to the
5 immobilized polynucleotide following washing. Washing ensures that the labelled polynucleotide is hybridized only to the immobilized polynucleotide with a desired degree of complementarity to the labelled polynucleotide.

It will be understood that polynucleotide homologues according to the invention will hybridise to a reference polynucleotide under stringent
10 conditions. Typical stringent conditions include, for example, (1) 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl at about 42°C for at least 30 minutes; or (2) 6.0 M urea/0.4 % sodium lauryl sulfate/0.1x SSC at about 42°C for at least 30 minutes; or (3) 0.1x
15 SSC/0.1% SDS at about 68°C for at least 20 minutes; or (4) 1x SSC/0.1% SDS at about 55°C for about 60 minutes; or (5) 1x SSC/0.1% SDS at about 62°C for about 60 minutes; or (6) 1x SSC/0.1% SDS at about 68°C for about 60 minutes; or (7) 0.2X SSC/0.1% SDS at about 55°C for about 60 minutes; or (8) 0.2x
20 SSC/0.1% SDS at about 62°C for about one hour; or (9) 0.2X SSC/0.1% SDS at about 68°C for about 60 minutes. For a detailed example, see CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at pages 2.10.1 to 2.10.16, and Sambrook *et al.* in MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbour Press, 1989) at sections 1.101 to 1.104, which are hereby incorporated by reference.

While stringent washes are typically carried out at temperatures
25 from about 42°C to 68°C, one skilled in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridisation typically occurs at about 20°C to 25°C below the T_m for formation of a DNA-DNA hybrid. It is well known in the art that the T_m is the melting temperature, or temperature at which two complementary polynucleotide sequences dissociate.
30 Methods for estimating T_m are well known in the art (see CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at page 2.10.8). Maximum

hybridization typically occurs at about 10°C to 15°C below the T_m for a DNA-RNA hybrid.

Other stringent conditions are well known in the art. A skilled addressee will recognize that various factors can be manipulated to optimize the specificity of the hybridization. Optimization of the stringency of the final washes can serve to ensure a high degree of hybridisation.

Methods for detecting a labelled polynucleotide hybridised to an immobilised polynucleotide are well known to practitioners in the art. Such methods include autoradiography, chemiluminescent, fluorescent and colorimetric detection.

4. Vectors

A polynucleotide according to the invention is suitably rendered expressible in a host cell by operably linking the polynucleotide with one or more regulatory nucleic acids. The synthetic construct or vector thus produced may be introduced firstly into an organism or part thereof before subsequent expression of the construct in a particular cell or tissue type. Any suitable organism is contemplated by the invention that may include unicellular as well as multi-cellular organisms. Suitable unicellular organisms include bacteria. Exemplary multi-cellular organisms include yeast, mammals and plants.

The construction of the vector may be effected by any suitable technique as for example described in the relevant sections of Ausubel *et al.* (*supra*) and Sambrook *et al.* (*supra*). However, it should be noted that the present invention is not dependent on and not directed to any one particular technique for constructing the vector.

Regulatory nucleotide sequences which may be utilised to regulate expression of the polynucleotide include, but are not limited to, a promoter, an enhancer, and a transcriptional terminator. Such regulatory sequences are well known to those of skill in the art. Suitable promoters that may be utilised to induce expression of the polynucleotides of the invention include constitutive promoters and inducible promoters.

5. Therapeutic agents

A further feature of the invention is the use of the polypeptide, fragment, variant or derivative of the invention ("*therapeutic agents*") as actives in a pharmaceutical composition for alleviating patients against blood loss.

5 Suitably, the pharmaceutical composition comprises a pharmaceutically acceptable carrier.

By "*pharmaceutically-acceptable carrier*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a

10 variety of pharmaceutically acceptable carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Any suitable route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Preferably,

15 20 an intravenous route is employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms

25 of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be

30 effected by using other polymer matrices, liposomes and/or microspheres.

Pharmaceutical compositions of the present invention suitable for

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more immunogenic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the immunogenic agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is therapeutically effective to alleviate patients from blood loss. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over time such as a reduction or cessation of blood loss. The quantity of the therapeutic agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the therapeutic agent(s) for administration will depend on the judgement of the practitioner. In determining the effective amount of the therapeutic agent to be administered in the treatment of blood loss, the physician may evaluate the progression of blood loss over time. In any event, those of skill in the art may readily determine suitable dosages of the therapeutic agents of the invention. Such dosages may be in the order of nanograms to milligrams of the therapeutic.

6. Anti-tumour agent

The invention also extends to an anti-tumour agent comprising a polypeptide, polypeptide fragment, variant or derivative according to the invention conjugated with an anti-fibrin antibody. Such a conjugate may be

to thereby inhibit progression and invasiveness of such tumours. Reference may be made in this regard to an abstract by Raut and Gaffney (1996, *Fibrinolysis* 10 (Suppl. 4):1-26, Abstract No 39) which is hereby incorporated by reference.

5 The anti-fibrin antibodies may include any suitable antibodies that bind to or conjugate with fibrin, preferably human fibrin. For example, the anti-fibrin antibodies may comprise polyclonal antibodies. Such antibodies may be prepared for example by injecting fibrin into a production species, which may include mice or rabbits, to obtain polyclonal antisera.

10 In lieu of the anti-fibrin polyclonal antisera obtained in the production species, monoclonal antibodies may be produced using the standard method as for example, described in an article by Köhler and Milstein (1975, *Nature* 256:495-497) which is hereby incorporated by reference, or by more recent modifications thereof as for example, described in "CURRENT PROTOCOLS IN IMMUNOLOGY" (1994, Ed. J.E. Coligan, A.M. Kruisbeek, 15 D.H. Marguiles, E.M. Shevach and W. Strober, John Wiley and Son Inc. which is hereby incorporated by reference) by immortalising spleen or other antibody producing cells derived from a production species which has been inoculated with fibrin.

20 Preferred monoclonal antibodies which may be used to produce the anti-tumour agent of the invention include, but are not limited to, the anti-fibrin monoclonal antibodies disclosed by Tymkewycz *et al* (1993, *Blood Coagul. Fibrinol.* 4:211-221) which is hereby incorporated by reference or the monoclonal antibody described by Raut and Gaffney (1996, *supra*).

25 Also contemplated are anti-fibrin antibodies which comprise Fc or Fab fragments of the polyclonal or monoclonal antibodies referred to above. Alternatively, the anti-fibrin antibodies may comprise single chain Fv antibodies (scFvs) against fibrin. Such scFvs may be prepared, for example, in accordance with the methods described respectively in United States Patent No 5,091,513, European Patent No 239,400 or the article by Winter and Milstein (1991, *Nature* 30 349:293) which are hereby incorporated by reference.

Any suitable procedure may be used to conjugate the anti-fibrin

antibodies with a polypeptide, polypeptide fragment, variant or derivative according to the invention. For example, reference may be made to the 'zero-length' cross linking procedure of Grabarek and Gergely (1990, *Anal. Biochem.* 185:131-135), which is incorporated herein by reference.

5 In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

EXAMPLE 1

10 *Characterization Of Two Plasmin Inhibitors From Pseudonaja Textilis Textilis Which Inhibit Bleeding In An Animal Model*

MATERIALS AND METHODS

Materials

Pooled lyophilised *P. textilis* venom was obtained from Mr Peter
15 Mirtschin, Venom Supplies, Tanunda, South Australia. Venom was reconstituted in 0.05 M tris-HCl buffer pH 7.4, at 10 mg/ml and the solution was centrifuged (2,000 g for 30 min) before chromatography or analysis. Sephacryl S-300, Sephacryl S-100, con A-Sepharose and DEAE-Sepharose CL-6B were obtained from Pharmacia Uppsala, Sweden, and the synthetic chromogenic substrate S-
20 2251 was from Chromogenix, Mölndal, Sweden. A highly purified plasmin from Sanofi/Choay Laboratories (Paris) was used for some kinetic experiments. All other buffers and reagents were Analar grade.

Preparation of plasminogen and plasmin

Human plasminogen was purified from outdated pooled citrated
25 plasma using the affinity chromatography procedure described elsewhere (Deutsch and Mertz. 1970, *Science* 170:1095). Human plasmin was prepared from plasminogen by activation with urokinase-bound Sepharose 4B (Robbins, KC., 1978 "Plasmin" *In*: Handbook of experimental pharmacology. Markwardt F, ed.

Berlin: Springer 46: 317,) and calibrated against the International Standard for plasmin (77/558).

Plasmin Inhibitory Assay

The plasmin inhibitory assay was carried out essentially as described elsewhere (Friberger *et al.* 1978., Haemostasis 7:138). 900 μ L of 0.15 M tris-HCl, pH 7.4, 25 μ L (0.1 IU) of plasmin, 25 μ L of inhibitor were added to 50 μ L of substrate S-2251 (3.0 mM) and the residual plasmin was determined by continuous measurement of the absorbance of 405 nm in a Hitachi 557 recording spectrophotometer. A standard curve of plasmin activity was prepared using the International Standard (77/558).

Purification of Txln 1 and 2

We here describe for the first time purification procedures which allowed the isolation of two distinct forms of the Txln inhibitor. A Sephacryl S-300 column (5.0 x 95 cm) was equilibrated at 4°C with 0.1 M ammonium acetate buffer (pH 7.0) at a flow rate of 1 mL per minute. 500 mg of lyophilised *P. texilis* venom was reconstituted in 25 mL of column buffer, and following centrifugation at 10,000 rpm for 20 minutes, was applied to the column. 12 mL fractions were collected using an LKB fraction collector, and the eluate was monitored at 280 nm using an Altex dual wavelength in line UV detector. The pooled plasmin inhibitor fractions were concentrated using an Amicon stirred cell concentrator Model 402 with a YM 3 membrane and this concentrate was applied to the DEAE-Sepharose column. The DEAE-Sepharose column (2.5 x 12 cm) was equilibrated at 4°C with 0.05 M phosphate buffer (pH 8.0) at a flow rate of 1.0 mL per minute. Following the application of the concentrated plasmin inhibitor, the column was washed with buffer giving a non-bound protein peak with no plasmin inhibitory activity. A linear gradient of NaCl (0-0.5 M, 500 mL) was applied at a flow rate of 1.0 mL per minute in order to separate the two forms of Txln. The pooled plasmin inhibitors 1 and 2 (concentrated in the Amicon cell) were individually further purified on a Sephacryl™ S-100 column (2.5 x 95 cm) which was equilibrated with 0.05 M Tris-HCl, (pH 7.4). Fractions with the

highest plasmin inhibitory activity were pooled, concentrated and stored at concentrations of about 1 mg/mL (\square 143 μ M) in Tris buffered saline. Finally a trace contaminant was removed from Txln 1 and Txln 2 samples by application to a column of Con A-Sepharose (1 x 10 cm) equilibrated with 0.15 M Tris-HCl buffer (pH 7.4). The pooled and concentrated plasmin inhibitors were applied to this column at a flow rate of 1.0 mL per minute and the inhibitory activity was found in the wash peak.

The purity of Txln preparations was checked by reverse phase (RP) HPLC on a Waters C₁₈ μ bond pack column (0.6 x 30 cm) equilibrated with 0.05 % trifluoroacetic acid (TFA) in water and developed using a 0 to 70% acetonitrile gradient in 0.05% TFA. The chromatography was monitored at 214 nm and the gradient was developed over 60 minutes. Further check on purity was performed using Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Electrophoresis (PAGE) (Weber and Osborn, 1969. *J. Biol. Chem.* 244:4406) while the samples were prepared by a method which incorporates 4 M urea in the sample solution (Gaffney and Dobos, 1971, *FEBS Lett.* 15:13).

Amino acid sequencing:

Reduction and carboxymethylation of Txln 1 and 2 were performed in 6 M guanidine hydrochloride, 0.1 M Tris-HCL buffer, 1 mM EDTA, (pH 9.5) with 10 mM dithiothreitol (DTT) for 2 hours under Argon at 37°C. The carboxymethylation (CM) step was performed with 15 mM iodoacetic acid for 30 minutes. The CM Txln 1 and 2 were digested with endoproteinase Lys C and endoproteinase Asp N respectively in 50 mM phosphate buffer, pH 8.0 at 37°C for 18 hours, using an enzyme to substrate ratio of (1:100). The reactions were stopped by acidification with TFA and the digests were fractionated by RP-HPLC on a Vydac C₈ column (2.1 x 150 mm) using a Hewlett Packard 1090 liquid chromatograph equipped with a diode-array detector. At a flow rate of 0.2 ml/min linear gradients were formed between 0.1% TFA in water and 0.1% TFA in 70% acetonitrile. All chromatographies were carried out at room temperature. Amino acid sequence determinations were carried out on a Hewlett Packard G10005A sequencer by first carrying out a long N-terminal sequencing of both

Txln 1 and 2. The C-terminal sequences for Txln 1 and 2 were derived from the C-terminal fragment obtained from endoLys C and endoproteinase Asp N digestions. The evidence for the sequence is derived from a long N-terminal sequence run of the whole molecule, an extended sequence of an endoLys C peptide obtained by further chromatography of one of the peptides isolated by reverse-phase chromatography and the sequence of an endoproteinase Asp N peptide. The C-terminal two amino acids were identified from the full-length c-DNA sequence obtained during the cloning and expression of textilinin in *E. coli* (as hereinafter described in EXAMPLE 2).

10 *Mass spectrometry*

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was performed with a Bruker Reflex mass spectrometer (Bruker-Franzen Analytik GMBH, Bremen, Germany) operated exclusively in the reflectron mode. Samples were diluted in 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid and 2 mL of a matrix comprised of 2,6-dihydroacetophenone containing diammonium hydrogen citrate prior to deposition of 0.5 -1 mL onto a stainless steel target.

Mouse tail vein bleeding model

A bleeding model was established using mature outbred Quackenbush mice (average 20 gram) of both sexes after anaesthesia was induced by intra peritoneal injection of 0.4 mL of a one in ten dilution of an equal volume mixture of Ketamine (100 mg/mL) and Rompun (xylazine, 40 mg/mL). Tail vein intravenous delivery of aprotinin, the two txlns (100 µg/100µL of saline for each substance) was performed after anaesthesia was established and tail excision was performed 2 minute later for each mouse. The dose of the plasmin inhibitors used in these experiments were similar to that used during human CPB surgery adjusted to the mouse weight of 20 grams. Blood loss was measured by collection into preweighed eppendorf tubes. Accuracy dictated that blood loss was measured by weight rather than volume. All mice were euthanized by cervical dislocation. All mice experiments were approved by the Ethics

Committee of the Princess Alexandra Hospital. This Committee did not encourage a dose-response study and the inventors consider that an adjusted dose used in human surgery was a realistic basis for these initial studies. Such a dose of the Txlns was observed not to induce any adverse effects on the mice when
5 observed over a period of 2 days.

Kinetics of plasmin inhibition

Procedures for investigation of plasmin inhibition kinetics by the two purified textilinins (Txln 1 and Txln 2) were in accordance with that described elsewhere (Stone *et al.*, 1984, *Biochim. Pharmacol.* 33:175) and differed
10 from the method used to study the impure Txln preparation (Willmott *et al.*, 1995, *supra*) in that 4-fold and 36-fold higher enzyme concentrations were used. This latter approach allowed truncation of time scale from one hour to ten minutes or less. Enzyme-inhibitor assays were performed at 25°C in 0.1 M Tris/HCl, pH 7.4, containing 0.01% (v/v) Tween 80. A concentration of either 2 nM or 18 nM
15 plasmin was used in these experiments with 75 µM chromogenic substrate (S-2251) and 16-410 nM Txln. On the grounds that the pattern of plasmin inhibition was of the form associated with slow tight-binding inhibition, the progress curves were analysed in terms of the relationship:-

$$[P] = v_s t + (v_s - v_o) \{1 - \exp(-kt)\} / k \quad (\text{Eq. 1})$$

which describes the time dependence of the concentration of chromogenic product [P] as a function of the initial (v_o) and ultimately attained (v_s) velocities and the apparent rate constant (k) for the transition between the initial and final (steady) states. For the present system the initial rate in experiments conducted with a fixed concentration of chromogenic substrate [S] exhibited no dependence upon inhibitor concentration [I] - a simplifying
25 circumstance that allowed v_o to be identified as the initial velocity in the absence of plasmin inhibitor (see equation 2). Under those conditions the rate constant (k) may be expressed in terms of the competitive inhibitor constant (K_I) and the Michaelis constant for chromogenic substrate (K_m) as

$$k = k_d [1 + [I] / \{K_I (1 + [S] / K_m)\}] \quad (\text{Eq. 2})$$

where k_d is the rate constant for dissociation of the plasmin-inhibitor complex (Stone *et al.*, 1984, *supra*). Since the steady-state velocity, v_s , may be expressed in terms of the maximal velocity V and the relationship for classical competitive inhibition, namely,

5
$$v_s = V[S] / \{ [S] + K_m(1 + [I]/K_i) \} \quad (\text{Eq. 3})$$

the inhibitor constant K_i and the dissociation rate constant k_d were the two curve-fitting parameters to emanate from global analysis of the progress curves.

RESULTS

10 Purification data

Figure 1(a) shows the Sephacryl S-300 chromatographic separation of proteins from the crude venom showing three major and two minor peaks of protein, labelled 1-5. Plasmin inhibitory activity is indicated in the right-hand shoulder of peak four (see shaded area), using the plasmin neutralisation assay to
15 monitor the eluted fractions. Further fractionation of the pooled inhibitor fractions, (Amicon YM3 concentrated), was performed on a DEAE-Sepharose CL-6B column. Figure 1(b) shows the resultant separation, indicating two distinct peaks of plasmin inhibitory activity, marked by solid horizontal bars and labelled 1 and 2. Each peak was pooled separately, concentrated and applied to a
20 Sephacryl S-100 column to remove trace impurities. Figure 2 shows the elution profile of Txln 1, which is identical to that of Txln 2, however the insert in Figure 2 shows the reverse-phase HPLC profiles of each Txln indicating each to have a distinct elution volume from this column. The purity of the Sephacryl S-100 eluted material was further demonstrated by SDS-PAGE gel electrophoresis (data
25 not shown). The final concentrated plasmin inhibitors were stored at -20°C in 0.05 M Tris buffered saline at a final concentration of about 1 mg/mL.

While these preparations were adequate for kinetic and physical characterisation, it was noted that both Txln 1 and 2 caused distress in the mouse model used to assess blood loss. For such experiments it was necessary to
30 remove trace amount of a potent prothrombin activator complex using a Con A-

Sephadex column as described elsewhere (Masci PP. 1986. *The effects of Australian snake venoms on coagulation and fibrinolysis*. Masters Thesis; University of Queensland).

Primary Sequence

5 Figure 3 shows the amino acid sequences of Txln 1 and 2 with those of aprotinin and Taicotoxin-associated plasmin inhibitor isolated from the venom of the Australian Eastern Taipan, *Oxyuramus scutellatus* (having the closest homology to Txln 1 and 2) for comparison. It can be seen that all four plasmin inhibitors have the cysteine arrangements that are typical of this group of plasmin inhibitors and endow them with great stability. It was found that Txln 1 and 2 could be heated at 80°C for two hours with no loss of inhibitory activity (unpublished data). A sequence difference of six amino acids was observed between Txln 1 and 2, while each showed, respectively, 45 and 43 % homology with aprotinin. There was 58% and 55% homology, respectively, between Txln 1 and 2 and the Taicotoxin associated plasmin inhibitor. Both Txlns are quite acidic proteins with nett negative charges of -4 (Txln 1) and -6 (Txln 2), while aprotinin is quite basic, having a nett charge of +6. Mass spectroscopy data for Txln 1 and 2 showed molecular weights of 6682.4 and 6689.3 (data not shown), which agreed quite well with the molecular weights from the amino acid compositions.

Kinetic data

Figure 4 presents progress curves for chromogenic substrate hydrolysis by 2 nM plasmin in the presence of 0-410 nM Txln 1. These data resemble more closely those reported for aprotinin (Willmott *et al.*, 1995, *supra*); our prior data with impure Txln did suggest simple competitive inhibition, whereas these latter data with purified Txln 1 and 2 resemble more the two-step mechanism of aprotinin. The inhibitor constant (K_i) deduced from those data by global analysis in terms of Equations 1-3 is presented in Table 3, together with corresponding values for Txln 2 and the mixture of textilins that co-chromatographed prior to DEAE-Sephadex chromatography.

TABLE 3

Plasmin Concentration		
	2 nM (n=6)	18 nM (n=6)
	Mean \pm SD	Mean \pm SD
Sephacryl 100 Pool (Txln 1 and 2)	$7.1 \times 10^{-9} \pm 0.2$	$13.9 \times 10^{-9} \pm 0.3$
Txln 1	$3.5 \times 10^{-9} \pm 3$	$2.6 \times 10^{-9} \pm 0.2$
Txln 2	$2.2 \times 10^{-9} \pm 0.2$	$2.8 \times 10^{-9} \pm 0.3$
Aprotinin*	5.3×10^{-11}	

Corresponding results from progress curves for experiments with a higher plasmin concentration (18 nM) are also summarised in Table 3. Comparison of the inhibitor constants for the isolated Txlns 1 and 2, which are indistinguishable from each other, with that for the partially purified preparation suggests that a 3- to 5-fold protein purification has been achieved by the ion-exchange and extra Sephacryl S-100 chromatography steps. The inhibitor constants shown in Table 3 are much smaller than the value of 150 μ M reported previously (Willmott *et al.*, 1995, *supra*) for the impure Txln preparation. The increased strength of Txln-plasmin binding observed in this present study presumably reflects the removal of unidentified compound(s) from the Txln during the later stages of the present more extensive purification procedure. Despite this, the K_i values of the pure Txlns for plasmin are about 100-fold less than that observed for aprotinin (Willmott *et al.*, 1995, *supra*).

Behaviour of Txlns in an animal bleeding model

Since Txln inhibition of plasmin activity is much weaker (100-fold, see Table 3) than that observed for aprotinin, an animal model has been used to establish the effectiveness of the Txln in stemming blood loss when it is used at the same dosage as aprotinin.

The effect of intravenous delivery of (tail vein) Txln 1 and 2 on the

blood-loss from an excised mouse tail vein is shown in Table 4 and for comparison the results for aprotinin are also shown.

TABLE 4

	Blood weight (gms) (N=24)	Average reduction in
	Mean \pm SD	blood loss (%)
Control (Saline)	0.869 \pm 0.245	-
Aprotinin (100 μ g)	0.352 \pm 0.152	59.5
Txln 1 (100 μ g)	0.386 \pm 0.250	55.6
Txln 2 (100 μ g)	0.329 \pm 0.234	62.2

5

The amount used was equivalent on a weight basis to the amount of aprotinin used clinically in humans and this was 100 μ g of each substance studied per average 20 gram mouse. It can be seen from Table 4 that aprotinin reduced blood loss by 60% while both Txlns reduced blood loss to a similar extent when compared with saline-injected controls. The validity of these comparisons may need further scrutiny as the amounts of the Txlns and aprotinin used in the animal model were based on plasmin neutralization in vitro and may be subject to some error. Molar comparison of amounts of these inhibitors to be used in future experiments may be more appropriate.

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DISCUSSION

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Reduction in blood flow during major surgery or following trauma is of current concern because of a deteriorating blood donor status. The increased incidence of viral contamination of blood has introduced socio-medical problems that do not seem to abate. There is anxiety concerning the contamination of blood by HIV, hepatitis B and C viruses, while the potential for cross-contamination by prions associated with Bovine Spongiform Encephalitis (BSE) and Creutzfeldt-

Jakob Disease (CJD) remains a major cloud over the whole blood transfusion area.

Aprotinin derived from bovine lung is used for the stemming of blood flow during surgical procedures such as cardio-pulmonary bypass (CPB) (Royston D. 1990. *Blood Coagul. Fibrinol.* 1:53; Royston D. 1992. *J. Cardiothorac. Vasc. Anesh.* 6:76,). Indeed, while CPB is the major surgical circumstance in which aprotinin is used, blood loss during neurosurgery (Gurdetti and Spallone. 1981. *Surg. Neurol.* 15:239), orthopaedic (Ketterl *et al.*, 1982. *Medizinische Welt* 33:480), liver (Neuhaus *et al.* 1989 *Lancet* ii:924) and urological (Kosters and Wand. 1973. *Urologe* 12:295) surgeries have been reduced using this drug. This widespread usage is despite some reports of thrombosis (Van der Meer *et al.*, 1996. *Thromb. Haemost.* 75:1; Cosgrove *et al.*, 1992. *Ann. Thorac. Surg.* 54:1031; Samama *et al.*, 1994 *Thromb. Haemost.* 71:663) and fatal anaphylaxis during cardiac surgery (Diefenbach *et al.*, 1995. *Anesth. Analg.* 80:830). While the exact mechanism of action of aprotinin is not known it is now accepted that plasmin inhibition is central to its capacity to reduce blood loss (Royston D. 1990., *supra*; Orchard *et al.*, 1993. *Br. J. Haematol.* 85:596). However, aprotinin has other effects on the coagulation cascade and on platelet function (Westaby, S. 1993. *Ann. Thorac. Surg.* 55:1033). The GPIIb/IIIa receptors which are mostly responsible for platelet adhesion are not affected by contact with bypass circuit surfaces whereas plasmin degrades the platelet GPIb receptor which can reduce the ability of platelets to form haemostatic plugs (Wenger *et al.*, 1989. *J. Thorac. Cardiovasc. Surg.* 97:235). Thus plasmin inhibition may also affect this latter platelet mechanism enhancing the stability of the haemostatic plug. It is worth while here to indicate that aprotinin has been found to inhibit protein C (Cooper BE. 1995. *J. Pharm. Technol.* 11:156), which in turn would result in reduction in thrombin production and enhanced fibrinolysis (Gaffney PJ, Edgell TA. Fibrinolysis and the haemostatic balance. "Harmonisation of some old and new concepts." *In: Recent progress in blood coagulation and fibrinolysis.* Takada A, Collen D, Gaffney PJ, Eds. Amsterdam; Elsevier Science BV 127, 1997).

Both these latter effects could reduce the effectiveness of aprotinins in reducing blood loss. While the lack of specificity of aprotinin leads

to confusion about its mechanism of action the inhibition of plasmin still seems to be central to its effectiveness. The reduction in the formation of the fibrin fragment D dimer in aprotinin-treated patients has been the main evidence (Orchard *et al.*, 1993, *supra*; Ray and Marsh. 1997. *Thromb. Haemost.* 78:1021; Dietrich *et al.*, 1990. *Anesthesiology* 73:1119) that plasmin inhibition is central to its mechanism; however it has been argued (Dietrich *et al.*, 1990, *supra*) that inhibition of fibrin formation and thus reduction in fibrin-mediated activation of plasminogen to plasmin could also offer an explanation for the reduction in D dimer levels.

In order to provide other alternative haemostatics based on plasmin inhibition, snake venoms have been studied for some years. The first report of a plasmin/trypsin inhibitor found in snake venom was by Takahashi *et al.* 1972. *FEBS Lett.* 27:207), while there are further reports of plasmin inhibitors in other viper and elapid venoms (Shafqut *et al.*, 1990. *Eur. J. Biochem.* 194 (2):337; Shajqut *et al.*, 1990. *FEBS Lett.* 275:6; Yamakawa *et al.*, 1987. *Biochim. Biophys. Acta* 925:124; Ritonja *et al.*, 1983. *Eur. J. Biochem.* 133: 427; Strydom *et al.*, 1979. *Biochim. Biophys. Acta* 491:361). Screening of Australian elapid venoms has shown that two snake genera possess potent plasmin inhibitors (Masci PP. Masters Thesis 1986, *supra*). These are the *Pseudonaja* and *Oxyuranus* genera. In the *Pseudonaja* genus, the venom from all species was shown to possess an inhibitor of plasmin. This inhibitor has been partially purified and kinetically characterised from the *textilis* subspecies (Wilmott *et al.*, 1995, *supra*) and has been subsequently named Textilinin (Txln). Further purification (Figures 1 and 2) has shown that there are two forms of this inhibitor, Txln 1 and 2. In the *Oxyuranus* genus, the venom of only one species was shown to contain a plasmin/trypsin inhibitor which has been sequenced and shown it to be associated in a multimeric complex (Possani *et al.*, 1992. *Toxicon.* 30:1343). This complex was demonstrated to be a calcium channel blocker containing an alpha neurotoxin, a phospholipase and the trypsin inhibitor called Taicotoxin. Figure 3 shows that this trypsin inhibitor (TAC) has 58 and 55% homology with Txln 1 and 2, respectively, and this is the closest homology to the Textilinins of the known naturally occurring plasmin inhibitors. There is only 45 and 43% homology between Txln 1 and 2,

respectively, and aprotinin. There are 6 amino acids difference between Txlns 1 and 2, and both are acidic, containing nett negative charges (-4 and -6 respectively), as distinct from aprotinin which is a basic molecule (+6).

While studying the kinetics of a partially purified plasmin inhibitor preparation from the *P. texilis* venom, it had been observed (Wilmott *et al.*, 1995, *supra*) that this inhibitor bound rapidly and more specifically to plasmin than did aprotinin (Fritz and Wanderer. 1983. *Drug Res.* 4:479). The results also showed that textilinin bound less tightly to plasmin than did aprotinin. The specificity of aprotinin was shown to be broad based, neutralizing tPA, urokinase and kallikrein, as well as plasmin and trypsin (Fritz and Wanderer. 1983, *supra*) while studies of the snake venom plasmin inhibitor, Txln, have shown it to bind more specifically to plasmin and trypsin in a rapid single step reaction which seems to be reversible (Wilmott *et al.*, 1995, *supra*). Since aprotinin has been reported (Van der Meer *et al.*, 1996, *supra*; Cosgrove *et al.*, 1992, *supra*; Samama *et al.*, 1994., *supra*.) to be associated with increased incidence of vein-graft occlusion and thrombosis, it was surmised that a less-tight binding inhibitor such as Txln may be of greater clinical efficacy. This original finding had prompted us to further purify the Txln from the venom and it was then found that each snake venom contained two forms of the Txln, which reflects the work of other workers (Takahashi *et al.*, 1974. *Toxicon.* 12:193) who also reported two variants of a Russell's viper venom plasmin inhibitor. Both Txlns bound to plasmin less tightly than aprotinin, but more strongly than has been indicated with partially purified material reported previously (Wilmott *et al.*, 1995, *supra*).

Txlns 1 and 2 reduce blood loss in a mouse tail-vein-bleeding model (Table 4) as effectively as aprotinin. If the reduction in blood loss in this model is associated with plasmin neutralisation at the site of the haemostatic plug formation as suggested (Royston D., 1992, *supra*), it is not surprising that they compare favourably. The inability of Txln to neutralise kallikrein in contrast to aprotinin (our unpublished data) may have some clinical significance. This, of course, depends on the contribution of the kallikrein-Factor XII pathway on the production of plasmin at the site of wound healing (Kluft *et al.*, 1987. *Sem. Thromb. Haemost.* 13:50). Indeed, the kallikrein inhibitory effect of aprotinin

could be a contributing factor to either a prothrombotic or prohaemorrhagic effect for this drug; the general opinion is that aprotinin inhibition of the extrinsic coagulation pathway via kallikrein-Factor XII would tend to inhibit coagulation following passage of blood through CPB machines (Westaby S., 1993, *supra*).

5 What role the Txln molecule plays in the human coagulation imbalance associated with this snake bite is unclear since envenomation is accompanied by a dramatically increased fibrinolytic activity which is, in turn, related to the disseminated intravascular coagulation in the bitten individual (Masci *et al.*, 1990. *Thrombosis Research* 59:859; Tibballs *et al.*, 1992. *Anesthesia and Intensive Care* 20:28). Presumably this fibrinolytic activity is stimulated by
10 the prothrombin-mediated fibrin complex (Gaffney and Edgel, 1997, *supra*). That the subsequent inhibition of fibrinolysis might contribute to this fibrin-mediated occlusion of the microvasculature is plausible.

 Currently it is the kinetic profile and the narrow specificity of the
15 Txlns that suggest strongly that there may be a clinical benefit over aprotinin to reduce blood loss. There is no doubt that the mouse bleeding model data indicate comparative blood loss reductions, but there are no physiological data suggesting that Txln may have less deleterious side effects than aprotinin. However, all mice treated with Txln showed no side effects. Notwithstanding this lack of evidence,
20 the fact that repeated therapeutic use of aprotinin is contra-indicated (Wüthrich *et al.*, 1992 *Lancet* 340:173) is sufficient to justify the cloning and expression of these new haemorrhagic inhibitors.

EXAMPLE 2

Cloning and Sequencing of Textilinin cDNA

25 MATERIALS AND METHODS

Materials

Common Brown Snake venom glands were obtained from reptiles deemed to be destroyed, having clinical conditions, which could not be treated. Venom glands were surgically taken, under sterile conditions, immediately after

the animals were euthanized by a lethal dose of pentobarbitone (60 mg/Kg). Department of Environment and Heritage as well as the University of Queensland Animal Ethics committee approved the termination of these reptiles. Two excised venom glands (approximately 100mg of wet tissue) were immediately frozen in liquid nitrogen and stored at -70°C until ready for total RNA extraction.

Degenerate primers

Masci-3 (sense) ATGAARGAYAGRCCHGARYTNGAR [SEQ ID NO:27];

Masci-5 (antisense) GTRCTYTCRTGYTCYTCY [SEQ ID NO:28];

Isolation of total RNA

Total RNA was isolated using the Dynal Bead total RNA extraction kit. Frozen venom glands (2) were placed in 1.0 mL of lysis buffer (supplied in the kit) in an Eppendorf™ tube and immediately homogenised using a RNAase-free sterile Polytron™ probe. Homogenisation was carried on ice in 4x 10-second intervals. The homogenate was divided in 0.5 mL aliquots and an equal volume phenol-chloroform (1:1) extraction carried out. The aqueous layer (top) was separated which contained RNA and DNA, which was precipitated with an equal volume of isopropanol overnight. After centrifugation at 13,000 rpm for 20 minutes at 4 °C, 70% ethanol washing was carried out. The precipitated RNA was reconstituted in DEPC-treated water and nucleic acid content determined on diluted aliquot by measurement of absorbance at 260 nm, using the formula:

$$\text{Total RNA (mg)} = A_{260} \times [0.04\text{mg} / (1 A_{260} \times 1 \text{ mL})] \times \text{dilution factor} \times \text{volume (mL)}.$$

Subsequent total RNA preparations were carried out using TRIzol™ reagent (Life Technologies) as per instruction manual. Briefly, 100 mg tissue was homogenised (using a Polytron™ homogeniser with the small homogenising attachment) in 1 mL of TRIzol™ reagent.

RNA analysis was carried out by electrophoresing a sample on a

denaturing formaldehyde agarose/EtBr gel. Mammalian total RNA showed typical two bright bands at 4.5 and 19 kb, these bands corresponds 28S and 18S ribosomal RNA. The ratios of intensity of these bands were approximately 2:1.

Isolation of mRNA

5 Messenger RNA was isolated using Dynal Magnetic Beads as recommended by supplier. After elution of mRNA from magnetic beads, 1 µg was used for reverse-transcriptase (RT) polymerase chain reaction (PCR) and the remainder was precipitated in one tenth volume of 3 M sodium acetate pH 5.2/ 2 volumes of absolute ethanol and stored at -70 °C.

10 *RT-PCR*

RT-PCR was carried using Promega RT kit MMLV-reverse transcriptase and the isolated total RNA (1 µg) and mRNA as template at 42 °C for 1.5 hours. The resulting cDNA was used for second strand synthesis. Second strand synthesis was carried out using T4 DNA polymerase, first strand cDNA as
15 template. The reaction was carried at 14 °C for 3 hours. Final volume of second synthesis reaction was 100 µL. Phenol-chloroform extraction was carried out and aqueous layer (top, containing double stranded cDNA) was transferred into a clean Eppendorf and cDNA was precipitated with ethanol overnight. After centrifugation at 13,000 rpm for 20 minutes at 4 °C precipitate was washed with
20 70% ethanol and reconstituted in 10 µL of sterile water and stored frozen at -20 °C until used in PCR amplification Txln cDNA using degenerate primers to Txln 1 and 2.

Amplification by PCR of Txln cDNA

 Sense and antisense degenerate oligonucleotide primers Masci-
25 3/Masci-5 were designed from the amino acid sequence of Txln1. Genomic DNA was isolated from the liver tissue of the Brown Snake and was also used as template in PCR using degenerate primers to determine the existence of any intron sequences in Txln cDNA.

 Using amplification parameters consisting of 94 °C/1minute; 46 °C

for 1 minute; 72 °C for 1 minute for 35 cycles, a PCR product of 177 base pairs was obtained corresponding to a polynucleotide encoding an expected 59 amino acids. Similarly, a 177 base pair product was obtained using genomic DNA. The 177 base pair PCR product was ligated into p-GEM 5zf and pGEX-2T, respectively. Resultant recombinant plasmids were used as templates for automated nucleotide sequence analysis. The respective nucleotide sequences encoding the mature polypeptides relating to *Txln 1* and *Txln 2* are shown in FIGS 6 and 7.

Preparation of pGEM-2T vector

pGEM-2T (Pharmacia-Biotech, about 5 pmol) was cleaved with *Bam*HI and *Eco*RI. The digestion products were fractionated by TAE-agarose gel electrophoresis and the linearised vector was purified using a QIAquick™ DNA extraction kit (QIAGEN) followed by ethanol precipitation.

Ligation

pGEM-2T or pGEX vector (0.3 pmol), and 1.5 pmol of 177 base pairs PCR product were added to a ligation mix containing 2 units of T4 DNA ligase in a total volume 30 µL. The ligation was carried out overnight incubation at 14 °C.

Transformation

Electroporation was performed with *E. coli* strain DH5α as host using one third of the ligation mixture (standard conditions). A total of not less than 10 “white” colonies were selected for each construct on indicator standard LB plates containing 0.1 mg ampicillin/mL. Six cDNA isomers were identified with specific designed primers and their sequences are presented.

Cloning was carried out using linearised pGEM-T-vector having a 3' terminal thymidine extending beyond each end of the linearised molecule (Promega Corporation; Cat No. A3600, Part No. A360A, Lot No. 96814). Purified *Txln* PCR product (prepared using Advantage2 Taq polymerase enzyme system (Clontech)) was ligated into these ends using T4 DNA ligase (Promega

Corporation). Recombinant plasmid containing Txln cDNA was then electroporated into *E. coli* DH5 α , and suitable transformants were selected using conventional blue/white selection criteria. At least 10 positive colonies were identified as containing the Txln cDNA PCR product (177 base pairs or full-length). Sequencing of Txln cDNA insert was carried out using dye terminator matrix (Clontech; Cat No. 403045) and submitted for sequencing using ABI Prism™ Model 377 sequencer.

Expression

At least ten colonies with good consensus sequences were selected and grown in 2YT medium in the presence of 100 μ g/mL ampicillin and 0.1 M IPTG to induce expression. Direct detection of fusion proteins was performed with 12% SDS-PAGE according to Laemmli, UK, (1970, *Nature* 277: 680).

Txln-GST fusion proteins were purified using affinity chromatography glutathione-Sepharose™ 4B (Amersham-Pharmacia Biotech; Cat No. 17-0756-01). Glutathione-Sepharose™ 4B gel was washed in PBS 4 times to ensure all thrombin inhibitors were removed before incubating with Txln-GST fusion proteins. Recombinant Txlns were cleaved from Txln-GST fusion protein bound to glutathione-Sepharose™ by incubating with thrombin (5U/mg of fusion protein) (Pharmacia-Biotech). For 1 mL of packed gel containing Txln-GST fusion proteins from 1 litre culture, 50 units of thrombin was added and incubated for 21 hours at room temperature. Supernatant samples were removed at 2, 7 and 21 hours and examined by SDS-PAGE for rec Txln.

Refolding of recombinant Txln

To maximise the efficiency of refolding of recombinant Txln, a combination of procedures was investigated as described for example by Bieri *et al.* (1995, *Biochemistry*, 34:13059-13065), which is incorporated herein by reference, and Norris *et al.* (1994. Aprotinin analogues and a process for the production thereof, US. Patent 5,373,090 to Novo Nordisk), which is incorporated herein by reference.

Briefly, recombinant Txln in 20 mM NH₄HCO₃, pH 8.3, with

added 2M guanidine hydrochloride was reduced with 45 mM DTT for 15 min
50 °C. The reduced and unfolded Txln was then quickly diluted by 100-fold (final
salt concentration is less than 0.05M) by adding to 20 mM ammonium
bicarbonate buffer, pH 8.3 and left to stand for 18 hours. Concentrating and
5 purification of active recombinant Txln (1-10 mg), was carried out by applying
the diluted Txln solution to DEAE-Sepharose™ (1.0 x 10 cm) ion-exchange
column as described for native Txln. Active recombinant Txln was assayed by
inhibition of plasmin (0.1 U), using S-2251 (3.0 mM) chromogenic assay. Clinical
efficacy of recombinant Txlns was investigated in mouse-tail vein bleeding
10 model.

RESULTS

cDNA Sequence of Textilinin 1 obtained using degenerate primers (Mascl-3/Mascl-5)

Primers (Mascl-3/Mascl-5) were designed based on codon
15 redundancy for amino acids and choosing specific regions of N-terminal and C-
terminal for Txln 1 and Txln 2 sequences (described below). Those were used to
amplify cDNA produced from total RNA isolated from the Brown snake venom
gland. The PCR products were cloned into pGEM-5zf(+) using blunt end cloning.
Positive clones (white) were further substantiated to contain the insert by PCR
20 screening, using Mascl-3/Mascl-5 as primers and plasmid DNA, prepared by
mini-prep procedure, as template. DNA sequence analysis using an ABI Dye-
terminator kit yielded two separate sequences for Txln 1 and Txln 2 (FIGS. 6 and
7). At least 10 separate clones were employed to obtain these sequences.

Design of gene-specific primers to determine the 5' and 3' Untranslated Regions (UTRs) of Txln cDNA

A new set of primers (F1 and R1; Txln2R1) was designed with two
nucleotide changes to increase the G-C content and thus the alignment of primer
to DNA. The two changes were in codon 6; TTT is changed to TTC (maintaining
code for F) and in codon 5; GAT is changed to GAC (again, maintaining the same
30 amino acid, D). A new forward primer, F1 was designed having the sequence
below.

F1: *Txln* 1 Gene-Specific Forward Primer

ATATATGGATCCAAGGACCGGCCTGACTTC [SEQ ID NO:29]

*Bam*HI

5 In the case of the reverse primer, R1, codon AGT (encoding amino acid 59) was changed to TCA, conserving the amino acid, Serine (S) and again, increasing the GC content of the R1 primer. The codon GG(N) (encoding amino acid 58) was changed to a C to optimise binding of the primer to DNA. A corresponding reverse primer specific for *Txln* 2, R2, was also employed. The primer sequences are listed below:

10

R1: *Txln* 1 Gene-Specific Reverse Primer

AACGGGAATTCTCAGAGCCACACGTGCTTTC [SEQ ID NO:30]

*Eco*RI stop

15

R2 *Txln* 2 Gene-Specific Reverse Primer

AACGGGAATTCTCATGAGCCACAGGTAGACTC [SEQ ID NO:31]

*Eco*RI stop

20 (*Txln* 2 gene-specific reverse primer gave a positive PCR product, although it was not used).

Amplification products were separated by agarose gel electrophoresis and a 177 bp amplicon was purified using QIAquick™ PCR purification kit (QIAGEN). 1-2 µg purified *Txln*-cDNA PCR product was ligated into pGEM-2T-vector and sequencing carried out using a dye terminator kit (Perkin-Elmer Corporation note, August 1995). The nucleotide sequence of *Txln* cDNA enabled us to design a second set of *Txln* 1-gene specific primers to determine the 5' and 3' sequences of the gene (3' and 5' RACE methodology). Those primer sequences are given below and have been designated gene specific primers (TX1FN and TX1RN) to distinguish them from the initial set.

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5' and 3'-SMART™ RACE cDNA amplification (Clontech).

A fresh preparation of cDNA was prepared for each 5'- and 3'-RACE reaction. The SMART™ RACE kit includes a protocol for the synthesis of two separate cDNA populations: 5'-RACE Ready cDNA and 3'-RACE Ready cDNA. The cDNA for 5'-RACE was synthesised using a modifying lock-docking oligo (dT) primer and the SMART™ II oligo. The modified oligo (dT) primer, termed 5'-RACE cDNA Synthesis primer (5'-CD's), has two degenerate oligo positions at the 3' end. These nucleotides position the primer at the start of the poly A+ tail and thus eliminate the 3' heterogeneity inherent with conventional oligo (dT) priming (Borsen *et al*, 1994, *PCR Methods Applic.* 2:144-148).

The 3' RACE cDNA was synthesised using conventional reverse transcription procedure, but with a special oligo (dT) primer. This 3'-RACE cDNA Synthesis (3'-CD's) primer includes the lock-docking nucleotide positions as in the 5'-CD's primer and also has a portion of the SMART™ sequence at its 5' end. By incorporating the SMART™ sequence in both the 5' and 3'-RACE-Ready™ cDNA populations, one can prime both RACE PCR reactions using the Universal Primer Mix (UPM), that recognises the SMART™ sequence, in conjunction with distinct *Txln* gene-specific primers. The primer set used for RACE is as follows:

Universal Primer mix:

Long Universal Primer (0.2 µM),

CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
[SEQ ID NO:32];

Short Universal Primer (1 µM),

CTAATACGACTCACTATAGGGC [SEQ ID NO:33];

Nested Universal Primer (NUP; 10 µM),

AAGCAGTGGTAACAACGCAGAGT [SEQ ID NO:34].

FIG. 8 shows the agarose gel electrophoretic mobility patterns of PCR products obtained with *Txln* gene-specific primers. PCR products (both 5'-

and 3'-RACE) were electrophoresed, excised and gel purified using QIAquick™ gel extraction kit (QIAGEN).

Cloning of region coding for proform of Txln 1

From 5' and 3' RACE sequences, *Txln*-gene specific forward (TX1FN) and reverse (TX1RN) primers were designed, containing a *Bam*HI restriction site in TX1FN (first 12 nucleotides) and an *Eco*RI site in TX1RN (12 nucleotides). The sequences for these primers are listed below:

TX1FN

10 ATCAGCGGATCCATGTCTGGAGGT [SEQ ID NO:35];

TX1RN

TCTCCTGAATTCTCAGGCAGCACAGGT [SEQ ID NO:36].

15 PCR was carried out using cDNA as a template and Advantage2™ Taq polymerase with the following conditions: 92 °C/1 min; 50 °C/1min; 72 °C/1 min for 30 cycles. These primers amplified a product corresponding to a sequence coding for the Txln1 proform (83 amino acids).

Cloning of Txln 1 proform

20 All three PCR products were purified from the gel and cloned into pGEM-2T for DNA sequencing using pGEM specific primers adjacent to the insert. The nucleotide and deduced amino acid sequences outlined in FIG. 9 [SEQ ID NO: 43 and 44, respectively] were derived by sequencing the 3' and 5' RACE products. This allowed the identification of an extra 72 nucleotides
25 upstream of the AAG (K) in frame, suggesting the presence of a proform of Txln1 existed. An extra 24 amino acids exists immediately upstream of the coding 59 amino acids. Eleven (11) nucleotides of 5' UTR was also identified as well as 143 nucleotides of 3' UTR. In addition 3' RACE sequencing revealed that the two amino acids immediately upstream from the stop codon were not alanines,
30 not glycine and serine as derived from the original less accurate sequencing. However, additional sequences to *Txln 1* and *Txln 2* were obtained by sequencing

multiple clones. After extensive sequencing, it became apparent that there were six separate *Txln* genes.

Cloning for the coding region of Txln1

Similarly, *Txln* gene-specific primers were designed to obtain a
5 PCR product, which encoded the active peptide (59 amino acids). Again, in this case, a *Bam*HI site was incorporated into the forward primer (TX1TF) and the reverse primer was the RACE-Ready Universal primer (Long SMART™).

Txln-active peptide sequence primers:

10

TX1TF (forward),
ATTATAGGATCCAAAGGACCGTCCGGAT [SEQ ID NO:37];

RACE-Ready long Universal primer

15 CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT
[SEQ ID NO:32].

Cloning of additional Txln genes

Forward primers were also designed for *Txln* 2-6 (below), and in combination with long Universal Primer (LUP, Clontech RACE-Ready Kit),
20 using the PCR conditions as described above. The sequences for these primers are as follows:

Forward primer for *Txln* 2 (TX2T)

ATTATAGGATCCAAAGGACCGTCCAGAG [SEQ ID NO:38];

25

Forward primer for *Txln* 3 (TX3T)

AACGTCGGATCCAAAGGACCGTCCAAAT [SEQ ID NO:30];

Forward primer for *Txln* 4 (TX4T)

30 AACGTCGGATCCAAAGGACCAATCCAAAA [SEQ ID NO:40];

Forward primer for *Txln 5* (TX5T)

AACGTCGGATTCAAAGGACCGTCCAAAA [SEQ ID NO:41]; and

Forward primer for *Txln 6* (TX6T)

5 ATTGTCGGATCCAAAGGACCTGCCAAAG [SEQ ID NO:42].

In all cases, the forward primer had a *Bam*HI site inserted to facilitate cloning. The underlined sequence marks the start triplet for the coding sequence.

10 Amplification products obtained using the above primers were fractionated by agarose gel electrophoresis and DNA fragments with the appropriate size were purified, and cloned into pGEM-2T vector. Sequencing of recombinant plasmids was performed using a Clontech dye terminator matrix and an ABI Prism™ Model 377 sequencer. Nucleotide sequences obtained by this
15 procedure for *Txln 1-6* are presented in FIG. 10 together with the corresponding deduced amino acid sequences. As will be apparent from inspection of FIG. 11, the *Txln* amino acid sequences are highly homologous and in this regard, a consensus sequence is provided.

20 *Recloning of Txln cDNA gel purified PCR product into pGEX-2T Expression vectors*

Recombinant *Txln* (both 59 amino acid peptide and 83 amino acid molecule containing 24 amino acid propeptide) were expressed using pGEX-2T constructs. Recombinant *Txln* activity was assayed by using the chromogenic substrate S-2251 and enzyme plasmin (Friberger *et al*, 1978). SDS-PAGE and
25 Western blotting using polyvalent antibodies to *Txln* identified recombinant *Txln* (FIG. 12).

EXAMPLE 3

Production of a fibrin-specific monoclonal antibody-textilinin 1 conjugate

30 A fibrin specific monoclonal antibody, MAb 12B3.B10 (IgG2A/kappa) (Tymkewycz *et al*, 1993, *supra*), will be chemically conjugated

with the plasmin inhibitor Txln 1 by a two step zero length crosslinking procedure according to Grabarek and Gergely (1990, *Anal. Biochem.* 185:131-135). Briefly Txln 1 will be incubated with a water soluble carbodiimide (EDC) in the presence of N-Hydroxysuccinimide (sulfo-NHS), and will result in the conversion of the carboxyl groups of Glu or Asp into succinimidyl esters. After removing excess EDC by gel filtration MAb 12B3.B10 will be added to the activated Txln 1. Crosslinking will result from nucleophilic substitution of the lysine-amino groups of the IgG for the succinimidyl moieties during a 2h incubation. The IgG-Txln 1 conjugate will then purified from free Txln 1 via size exclusion HPLC on a Superdex 200 HR 10/30 column as described by Raut and Gaffney (1996, *Fibrinolysis* 10 (Suppl. 4):1-26, Abstract No 39). The purified construct will then be tested for plasmin inhibitory activity by ELISA using the Chromogenic substrate S-2251.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appendant claims.

TABLE LEGENDS

TABLE 1. *Conservative amino acid substitutions*

5 TABLE 2. *Unconventional amino acids for generation of modified peptides.*

TABLE 3. *Summary of inhibitory constants.* K_i for Txln S-100 Pool measured using Enzfitter analysis programme, using plasmin concentration 0.5 nM, was 0.15 μ M (n = 6). *Denotes data obtained from previous work
10 (Willmott *et al.*, 1995, *supra*) where the concentration of plasmin was used to determine K_i for aprotinin was 0.5 nM.

TABLE 4. *Mouse tail bleeding model - Blood loss determination.* The blood loss in the mice treated with aprotinin and the two forms of Txln (1 and 2)
15 compared to a saline control group is shown, while the percentage reduction in blood loss is also given.